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**PROFILE ANALYSIS AND IN VITRO ABSORPTION  
STUDY OF CITRUS PEEL POLYMETHOXYFLAVONES**

**by**

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Dr. Chi-Tang Ho

And approved by

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# **ABSTRACT OF THE THESIS**

## **Profile Analysis and In Vitro Absorption Study of Citrus Peel Polymethoxyflavones**

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Polymethoxyflavones (PMFs) are a group of flavonoids found exclusively in citrus genus that have been identified with many potent biological activities, including anti-inflammation, anti-cancer, anti-atherosclerosis, and antioxidant. However, the bioavailability of PMFs is seldom studied. As far as we know, PMFs have poor aqueous solubility, and may potentially have high permeability due to their lipophilic structure.

Biological studies rely on efficient separation method to obtain pure compounds. Silica gel column chromatography was utilized in this study to separate individual PMFs from aged orange peel extracts, and the yielded products had more than 95% purity. With this established method, purified PMF derivatives from chemical modification were also obtained.

In this research, we systemically investigated permeability and solubility of totally six PMFs and their derivatives, namely, sinensetin (SIN), 5-hydroxy-sinensetin (5-OH-SIN), 5-acetyl-sinensetin (5-Ac-SIN), 3,5,6,7,8,3',4'-Heptamethoxyflavone (HeptaMF), 5-hydroxy-3,6,7,8,3',4'-hexamethoxyflavone (5-OH-HeptaMF), and 5-acetyl-3,6,7,8,3',4'-hexamethoxyflavone (5-Ac-HeptaMF). Among them, 5-OH-SIN, 5-Ac-SIN, and 5-Ac-HeptaMF were prepared via chemical modification. From the octanol/water solubility test, it was found that PMFs and their derivatives had very low aqueous solubility. Permeability experiment via Caco-2 cell monolayer transport model was conducted indicating high permeability of all the tested compounds. Furthermore, PMFs had the greatest permeability, followed by 5-acetyl PMFs and 5-hydroxy PMFs. Moreover, metabolism especially deacetylation was observed during the transport of 5-acetyl PMFs. Considering permeability and solubility together, PMFs and their derivatives are expected having good absorption. This study gives indicative information on the bioavailability of PMFs and their derivatives, which provides clues on the application of PMFs in functional food or nutraceutical products.



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## **Chapter 1 Introduction**

### **1.1 Polymethoxyflavones**

#### **1.1.1 Origins**

In recent years, bioactive compounds gradually become popular due to many potential health benefits. Flavonoids, as a group of polyphenolic compounds originated from plant sources, have been proved having powerful biological functions. A plenty of flavonoids can be found in citrus, both in the juice and peels. Previous studies have revealed the flavonoids composition of different citrus plants. For example, lemon is rich in flavanones (eriocitrin and hesperidin) and flavones (apigenin 6,8-di-C-glucoside and diosmetin 6,8-di-C-glucoside) in the juice, pulp, and peel with different concentrations (Russo et al., 2014). Hesperidin is the predominant flavonoid in orange juice, similar as naringin in grapefruit juice (Bronner & Beecher, 1995; Zhang, Duan, Zang, Huang, & Liu, 2011).

According to the newest report on world citrus market from the United States Department of Agriculture (USDA, 2015), the global orange production was reduced to 48.8 million metric tons for the year of 2014/15 with 19.4 million metric tons for processing, generating a large volume of peels as the by-product; meanwhile, the global production of tangerine/mandarin is increased to 27.0 million metric tons, where 68% of the total production is from China. In China, tangerine/mandarin are consumed mainly as fresh

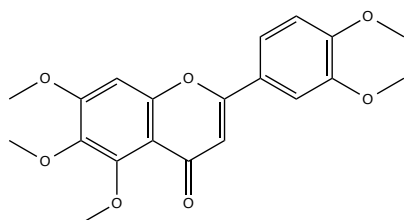


fruit, and a small portion of generated peels are collected and dried over sunshine. The aged tangerine/mandarin peels are called Chenpi, which has been used as traditional medicine as well as seasoning in China for thousands of years. As the traditional medicine, Chenpi has been used to treat stomach upset, cough, skin inflammation, muscle pain, and ringworm infections.

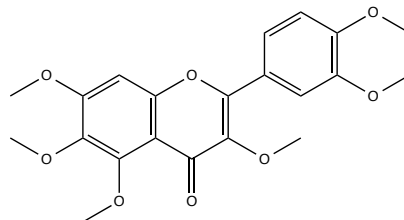
### **1.1.2 Major Polymethoxyflavones and Hydroxylated Polymethoxyflavones**

Polymethoxyflavones (PMFs) are found almost exclusively in citrus genus, especially in the peels of sweet orange (*Citrus sinensis*) and mandarin (*Citrus reticulata* Blanco).

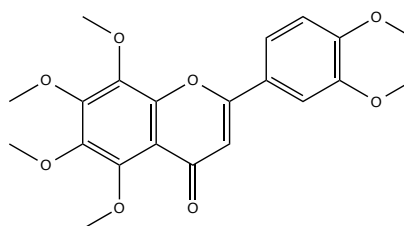
Up to date, there are more than 20 polymethoxylated flavonoids, which have been isolated and identified from different tissues of citrus plants (Li, Lo, Dushenkov, & Ho, 2008). Among them, there are six major PMFs (Figure 1.1), namely sinensetin (SIN), 3,5,6,7,3',4'-hexamethoxyflavone (HexaMF), nobiletin (NOB), 5,6,7,4'-tetramethoxyflavone (TetraMF), 3,5,6,7,8,3',4'-heptamethoxyflavone (HeptaMF), and tangeretin (TAN), which are all present in the aged orange peels. NOB and TAN are the two most abundant naturally occurring PMFs, followed by SIN. Subsequently, 5-demethyl-nobiletin (5-OH-NOB) and 5-demethyl-tangeretin (5-OH-TAN) are the two most common and abundant hydroxylated PMFs, which are resulted from auto-hydroxylation in the aged orange peels. Other hydroxylated PMFs, including 5-hydroxylated PMFs other than nobiletin and tangeretin, as well as hydroxylated at C3', C4', and C7 positions are present in trace concentrations. Hydroxylation at C3', C4', and C7 positions may be due to the action of microorganisms.



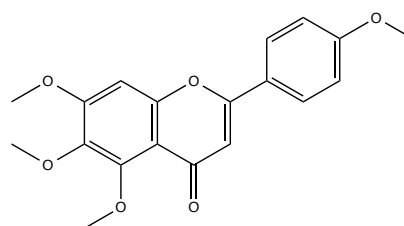
Sinensetin  
(5,6,7,3',4'-Pentamethoxyflavone)



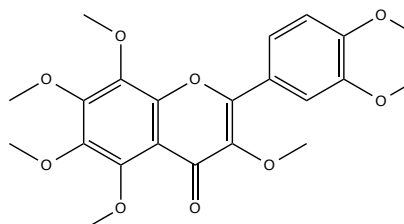
3,5,6,7,3',4'-Hexamethoxyflavone



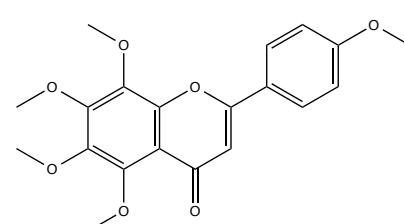
Nobiletin  
(5,6,7,8,3',4'-Hexamethoxyflavone)



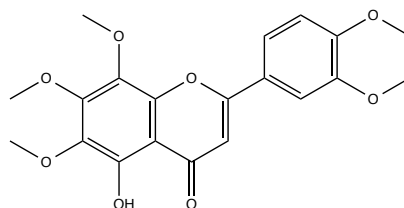
5,6,7,4'-Tetramethoxyflavone



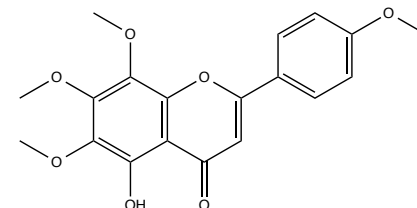
3,5,6,7,8,3',4'-Heptamethoxyflavone



Tangeretin  
(5,6,7,8,4'-Pentamethoxyflavone)



5- Demethyl-Nobiletin  
(5-Hydroxy-6,7,8,3',4'-Pentamethoxyflavone)



5- Demethyl-Tangeretin  
(5-Hydroxy-6,7,8,4'-Tetramethoxyflavone)

Figure1.1 Structures of major PMFs and 5-hydroxylated PMFs.

### **1.1.3 Health Effects of Polymethoxyflavones**

In Asian countries, like China and Japan, aged orange peel is used as traditional medicine for the treatment of stomach upset, cough, skin inflammation, muscle pain, and ringworm infections. Polymethoxyflavones, as the unique group of flavonoids in citrus peels, could potentially be the active ingredients accounting for the health benefits of aged orange peel. Therefore, numerous studies have been conducted on PMFs showing the evidence of their anti-inflammatory (Gossiau, Chen, Ho, & Li, 2014; Ho, Pan, Lai, & Li, 2012), anti-carcinogenic (Lai et al., 2007), antioxidant (Teng, Yang, Chen, Lin, & Tsai, 2011), anti-atherogenic (Kurowska & Manthey, 2004), hypolipidemic (Kurowska & Manthey, 2004) functions, and so forth. Unfortunately, the research on bioavailability of PMFs is on the way, which brought the significance of this presenting study.

### **1.1.4 Chemical Modification on Polymethoxyflavones**

As it is shown clearly from the chemical structures of PMFs, they all have poor aqueous solubility, which would greatly affect their bioavailability. From previous studies, it is said that some 5-OH-PMFs generally have better biological effects than their PMF counterparts (Li et al., 2009), such as HeptaMF. However, in reality, 5-OH-PMFs have even lower aqueous solubility than PMFs since the hydroxyl group tends to form a hydrogen bond with its adjacent ketone group.

In order to improve the solubility of 5-OH-PMFs, acetylation was proposed. 5-Acetyl-PMFs (5-Ac-PMFs) can be recognized as pro-drugs of 5-OH-PMFs and PMFs in the concept of pharmaceutical industry, meaning 5-Ac-PMFs are expected to convert into 5-OH-PMFs after administered. It was found previously that 5-Ac-TAN showed better cytotoxic effect than TAN and 5-OH-TAN in cell studies (Wang et al., 2014). With this in mind, the comparison on bioavailability of PMFs, 5-OH-PMFs, and 5-Ac-PMFs is of particular interest to better understand the difference in their biological effects.

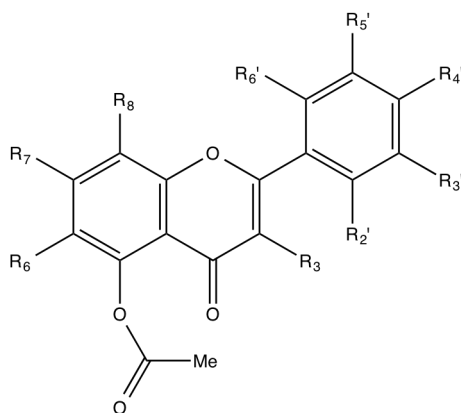


Figure 1.2 Structure of 5-Ac-PMFs.

## **1.2 Separation and Isolation Techniques**

### **1.2.1 Chromatographic-Analysis Techniques**

In the area of food chemistry, studies on PMFs have been focused on the analysis and identification of individual PMFs. Proposed methods include high performance liquid chromatography (HPLC) (Li, Lambros, Wang, Goodnow, & Ho, 2007; Wang, Li, Ferguson, Goodnow, & Ho, 2008), high performance liquid chromatography-mass spectrometry (HPLC-MS)/nuclear magnetic resonance (NMR) (Li et al., 2007; Wei et al., 2013), gas chromatography (GC) (Gaydou, Berahia, Wallet, & Bianchini, 1991), and GC-MS (Wei et al., 2013).

Among those techniques, HPLC analysis is the most common one for rapid detection of PMFs. A well-established HPLC method (Wang et al., 2008) was proposed using Supelco's RP-Amide C18 column and water (A)/acetonitrile (B) as the mobile phase for a 20 min gradient: 40% B at 0 min, then linearly increase to 55% B in 10 min, and then linearly increase to 70% in 15 min, finally stay at 80% at 20 min. This method was used throughout present study.

### **1.2.2 Isolation Techniques**

Most of the research papers on PMFs discuss the biological functions based on in vitro studies. In vivo study on PMFs is limited by the lack of large quantity of individual

compounds. Old-fashion isolation of PMFs relies mainly on silica gel column chromatography, which can isolate both large and small quantities. However, this method needs intensive labor and time, and the purity of isolated compound varies. PMFs such as nobiletin and tangeretin, which have different chromatographic behaviors in both normal and reverse phase HPLC, would be easily separated; however, nobiletin and 5,6,7,4'-tetramethoxyflavone would not be separated on silica gel column due to their similar chromatographic behaviors. As technology develops, new techniques are established to replace traditional methods for a better isolation of natural compounds.

Preparative HPLC system using reverse-phase column is quite common in the separation of PMFs. However, this method is more suitable for isolation of small quantities; and the amount obtained is usually only enough for *in vitro* studies. Therefore, an isolation method that can handle large-scale production becomes more important.

Flash chromatography is an upgraded method to fast separate natural compounds by applying pressure from the top of the silica column. While silica gel is the traditional material for flash chromatography, the application of reversed-phase packing is becoming more popular. Detailed method for separating PMFs using automated flash chromatography system is described in previous literature (Uckoo, Jayaprakasha, & Patil, 2011), where hexanes/acetone system was used as the mobile phase, and 5 g orange peel extract sample was loaded with a yield of 660 mg of nobiletin and 621 mg of tangeretin. Flash chromatography can be used for both large- and small-scale separation depending on the column size. One of the limitations of flash chromatography is the need of

excessive amount of organic solvents. The purity of PMFs is not guaranteed, and often time multi-cycle purification is required.

The principles for supercritical fluid chromatography (SFC) are similar to HPLC except for using carbon dioxide as the mobile phase. Compared to other separation methods, like normal phase chromatography, SFC method is more cost-effective and time-saving. SFC method with a mobile phase of 45% methanol and 55% supercritical CO<sub>2</sub> was reported to isolate large-scale PMFs (Li, Lambros, Wang, Goodnow, & Ho, 2007). In this study, DAICEL AD chiral column was found the best in the separation of PMFs on SFC after screening various columns, which is possibly due to the large amount of hydrogen bonds between methoxy groups of PMFs and hydroxyl groups of cellulose in the column. SFC system is able to separate PMFs with similar chromatographic behaviors, such as nobiletin and 5,6,7,4'-tetramethoxyflavone, as well as to achieve large-scale separation via either stack injection or SFC system with fast flow rate and large column.

In addition, all the isolation methods have to cooperate with other spectroscopic analysis, such as HPLC, LC-MS, and NMR, in order to identify the separated compounds and confirm their purities.

## 1.3 Bioavailability

### 1.3.1 Overview

In general, bioavailability of a drug or a nutrient is defined as the amount of the drug or the nutrient reaches the systemic circulation, which is the overall effect of absorption, distribution, metabolism, and excretion (Figure 1.3) (Ting, Jiang, Ho, & Huang, 2014).

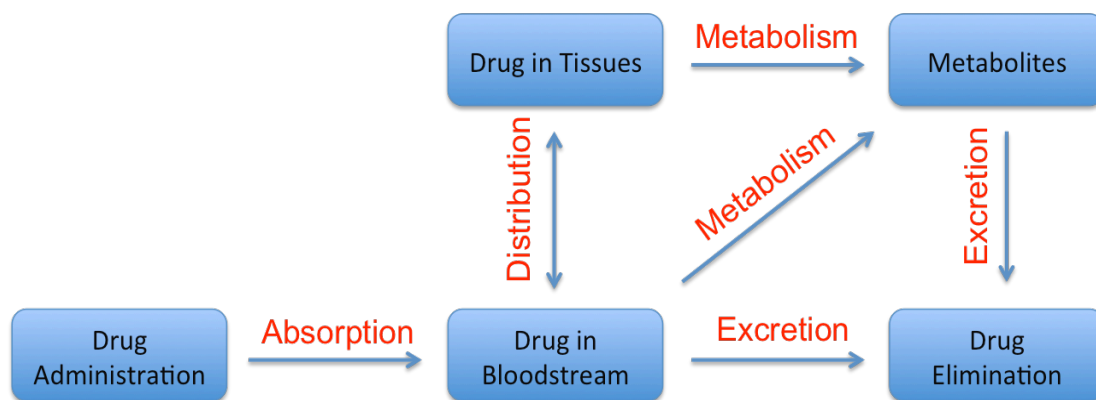


Figure 1.3 Absorption, distribution, metabolism, and excretion (ADME).

In order to obtain a whole picture of the biological functions for a certain nutrient, it is crucial to study its bioavailability first. As the first factor of bioavailability, absorption is affected by both solubility and permeability. Generally, the drug or nutrient firstly needs to be present in a solution when passing through the gastrointestinal tract, and then it can be picked up by the small intestine (Li, Wang, Ho, & Dushenkov, 2008). This presenting



study investigated the solubility and permeability of PMFs, and details will be discussed in the following chapters.

### **1.3.2 Bioavailability of Flavonoids**

Previous research has proved the health benefits of various polyphenols from plant sources. The bioavailability tests of these natural compounds were carried out by either in vivo measuring plasma concentrations or in vitro through Caco-2 monolayer transport model.

For polyphenols, poor bioavailability is resulted from the free hydroxyl groups, which are the targets of conjugations, like glucuronidation and sulfation (Manach, Williamson, Morand, Scalbert, & Rémésy, 2005). For example, quercetin, as a flavonol with five hydroxyl groups, is absent from plasma in the form of pure compound, but exists only in its conjugated forms (Manach et al., 2005). It was reported that methylated flavones showed 5 to 8 fold higher permeability than unmethylated flavones from the Caco-2 monolayer transport model (Wen & Walle, 2006), which indicates that the bioavailability is improved by methylating the hydroxyl groups. This statement has been proved by other studies as well. By comparing (–)-epigallocatechin-3-O-gallate (EGCG) with its O-methyl derivatives, it was found that EGCG3”Me showed higher bioavailability than EGCG in rat model (Oritani et al., 2013). Similarly, PMFs are expected to have high bioavailability as well due to the multiple methoxyl groups.

### **1.3.3 Bioavailability of Polymethoxyflavones**

The bioavailability of PMFs has rarely been reported, which makes present study stand out for its systemic comparison of PMFs and their derivatives on bioavailability. It was previously reported that nobiletin showed 48.1% permeability from apical to basolateral side in a Caco-2 monolayer transport study during a 4-hour incubation with a preference of accumulating in the monolayer (Murakami et al., 2001). Furthermore, by testing on the SD male rat, nobiletin had a wide distribution and accumulation in organs including stomach, small intestine, large intestine, liver, and kidney after 4 hours of a single dose treatment (Murakami et al., 2002). Another study showed that the overall solubility of PMFs is low, however, it was found that the permeability of 3,5,6,7,8,3',4'-heptamethoxyflavone, 3'-hydroxy-5,6,7,4'tetramethoxyflavone, and 3-hydroxy-5,6,7,8,3',4'-hexamethoxyflavone through the Caco-2 monolayer were excellent (Li et al., 2008). These previous studies lead to the hypothesis that PMFs could potentially have high bioavailability.

### **1.3.4 Caco-2 Cell Monolayer Transport Model**

As mentioned, permeability through small intestine is a crucial factor in the absorption process. In the small intestine, the monolayer of epithelial cells serves as a barrier to selectively absorb drugs or nutrients. For in vitro studies of permeability, the key is to find a substitute, which can mimic the function of human differentiated epithelial cells.

Caco-2, which is the human colon carcinoma cell line, was found perfectly fitting into this position.

In the permeability study, Caco-2 cells are cultivated onto permeable filters and gradually differentiate into a monolayer with tight junctions and apical (mucosal) side and basolateral (serosal) side during the 21-day incubation (Figure 1.3).

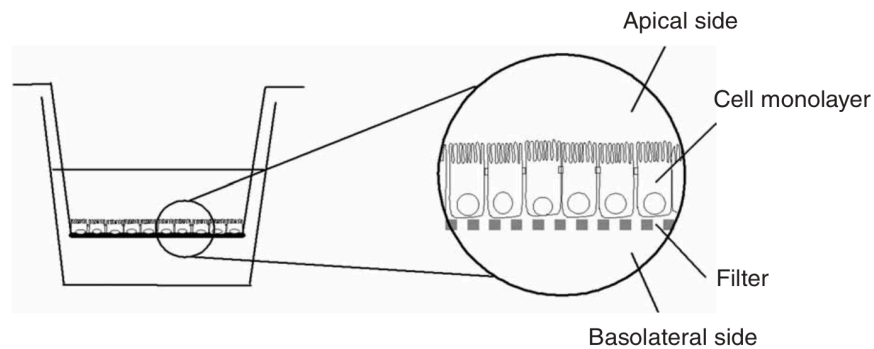


Figure 1.4 Diagram of Caco-2 monolayer on permeable filter (Hubatsch, Ragnarsson, & Artursson, 2007).

There are several facts regarding Caco-2 monolayer, which make it a good candidate to assess permeability:

- 1). Caco-2 monolayer has similar transporters and enzymes as epithelial cells:

On the apical side, efflux transporters were found including P-glycoprotein (Hunter, Jepson, Simmons, Hirst, & Tsuruo, 1993), multidrug resistance-associated protein 2 (Hirohashi et al., 2000; Prime-Chapman, Fearn, Cooper, Moore, & Hirst, 2004), and breast cancer resistant protein (Wright, Haslam, Coleman, & Simmons, 2011); and the absorptive transporters include organic anion transporting polypeptide (Nishimura et al., 2007) and proton-coupled amino acid transporter (Frolund, Langthaler, Kall, Holm, & Nielsen, 2012). On the basolateral side of Caco-2 monolayer, multidrug resistance-associated protein 3 is one of the major expressed efflux transporters (Prime-Chapman et al., 2004). Furthermore, metabolic enzymes are also expressed in Caco-2 cells, including sulfotransferases (Sato, Matsui, & Tamura, 2000) and glutathione S-transferases (Kusano, Horie, Morishita, Shibata, & Uchida, 2012).

## 2). Transport pathways:

There are four transport pathways, namely passive transcellular, passive paracellular, carrier mediated route, and transcytosis; and a drug moves across epithelial cells by one or more of these routes (Artursson, Palm, & Luthman, 2001). Generally, lipophilic compounds penetrate well through the membranes of brush border, which is the passive transcellular way; hydrophilic compounds and peptides mainly move slowly through the water-filled pores of paracellular route (Artursson et al., 2001).

## 3). The apparent permeability coefficient (Papp) and the efflux ratio (EfR):

Papp measures the amount of compound transported from one side of the Caco-2 monolayer to the other side per time. A compound has good permeability if  $P_{app} > 100 \times 10^{-7} \text{ cm/s}$  (Li et al., 2009). EfR is the ratio of  $P_{app}$  (B to A) to  $P_{app}$  (A to B), indicating the influence of efflux and absorption. Generally, if EfR is greater than 3, it means efflux liability is present (Li et al., 2009).

Caco-2 cell monolayer transport experiment gives important indicative information on the investigation of drug permeability through small intestine, and further to predict in-vivo drug absorption.

## **Chapter 2 Quantification on Polymethoxyflavones from Different Aged Orange Peel Extracts**

### **2.1 Principle**

Aged orange peel (also called Chenpi) is widely used in China as traditional medicine or seasoning for thousands of years. The price of Chenpi varies depending on the planting location as well as the year of storage. Chenpi from Sichuan province is inexpensive with \$10-20/Kg; however, Xinhui Chenpi from Guangdong province of China could cost as high as several thousand US dollars per kilogram. It is also said that the value of Chenpi increases with years of storage. Obviously, the interested components in Chenpi are polymethoxyflavones (PMFs), which have been proved having many health benefits. However, the price variations make researchers wonder if Chenpi from different sources could possibly make a big difference in the PMF composition.

In this study, the compositions of two Chenpi (one from Jiangxi province and the other one from Guangdong province) were analyzed using high performance liquid chromatography (HPLC). Traditional extraction methods on natural ingredients usually rely on large amount of organic solvents, and they are time consuming and laborious with low selectivity and extraction yields. The Chenpi samples used in this study were extracted by supercritical fluid extraction (Super-CFE) and sub-critical fluid extraction (Sub-CFE), which both can well extract lipophilic compounds due to the use of low-polarity fluids.

Between these two methods, Super-CFE using supercritical carbon dioxide is considered more promising on extracting PMFs since it has tremendous advantages over traditional methods. For example, supercritical carbon dioxide can fast penetrate and extract solid materials due to its low viscosity and high diffusivity; the selectivity of Super-CFE can be adjusted by changing the temperature and pressure; it generally gives a large yield; and it is more environmentally friendly because carbon dioxide is considered safer than other organic solvents and can be readily recycled (Herrero, Cifuentes, & Ibanez, 2006). Sub-CFE using mainly n-butane is also one of the most popular methods to extract natural compounds with many advantages including lowering temperature and pressure, time saving, good selectivity, environmental compatibility and so forth (Liu, Mei, Wang, Shao, & Tao, 2014).

By comparing the two Chenpi samples as well as the two extraction methods, we will get a rough idea about the Chenpi market, and at the same time how to extract to make the best of Chenpi product.

## **2.2 Experimental**

### **2.2.1 Materials**

The aged orange peel extracts were provided by Dr. Qingrong Huang (Food Science Department, Rutgers, the State University of New Jersey, USA). These samples were

prepared in the South China Agricultural University using two extraction methods: super-critical fluid extraction (Super-CFE) and sub-critical fluid extraction (Sub-CFE).

Sample 1: Chenpi from Jiangxi Province, China (Extracted by Super-CFE)

Sample 2: Chenpi from Jiangxi Province, China (Extracted by Sub-CFE)

Sample 3: Chenpi from Guangdong Province, China (Extracted by Super-CFE)

Sample 4: Chenpi from Guangdong Province, China (Extracted by Sub-CFE)

PMF standards including nobiletin (NOB), tangeretin (TAN), sinensetin (SIN), 3,5,6,7,3',4'-hexamethoxyflavone (HexaMF), 3,5,6,7,8,3',4'-heptamethoxyflavone (HeptaMF), 5,6,7,4'-tetramethoxyflavone (TetraMF), 5-hydroxy-6,7,3',4'-tetramethoxyflavone (5-OH-SIN), 5-hydroxy-3,6,7,3',4'-pentamethoxyflavone (5-OH-HexaMF), 5-hydroxy-6,7,8,3',4'-pentamethoxyflavone (5-OH-NOB), 5-hydroxy-6,7,4'-trimethoxyflavone (5-OH-TetraMF), 5-hydroxy-3,6,7,8,3',4'-hexamethoxyflavone (5-OH-HeptaMF) and 5-hydroxy-6,7,8,4'-tetramethoxyflavone (5-OH-TAN) were provided by Dr. Shiming Li (Food Science Department, Rutgers, the State University of New Jersey, USA).

Acetonitrile (HPLC grade), water (HPLC grade), and methanol (HPLC grade) were purchased from Pharmco-AAPER (Brookfield, CT, USA).

### **2.2.2 Apparatus**



The HPLC system consisted of a Dionex UltiMate 3000 HPLC series (Sunnyvale, CA, USA) including an UltiMate 3000 Pump, an UltiMate 3000 Variable Wavelength Detector, and an UltiMate 3000 Auto-sampler. Chromeleon software was used to perform instrument control and data analysis. Supelco Ascentis® RP-Amide C18 HPLC column (15 cm X 4.6 mm, 3 µm) was purchased from Sigma-Aldrich (St. Louis, MO, USA).

### **2.2.3 Method**

The orange peel extract (0.025 g) was dissolved in methanol within a 50 mL volumetric flask, which ended up with the concentration of 0.5 mg/mL. Sonication was applied if dissolved poorly. After filtration with 0.45 µm PTFE filter, the samples were ready for HPLC injection. The HPLC analysis for each sample was repeated three times.

The HPLC program used for PMFs is as following:

The mobile phase consists of water (A) and acetonitrile (C); and the 20 min gradient starts with 40% C, then linearly increases to 55% C in 10 min, and then linearly increases to 70% C in 15 min, finally stays at 80% C at 20 min; then increases to 100% C in 21 min and keeps there for 4 min to wash the column, and then goes back to 40% C for equilibrium. Data was collected at wavelength of 214 nm, 254 nm, and 326 nm. The injection volume was set at 5 µL, and the flow rate was 1 mL/min.

The HPLC program used for 5-OH-PMFs is as following:

The mobile phase consists of three solvents, namely, water (A), mixture of 80% acetonitrile and 20% tetrahydrofuran (B), and acetonitrile (C). The gradient starts with 35% B, then linearly increases to 45% B in 15 min, and then linearly increases to 50% B in 25 min; at 25 min, starts to introduce C, which linearly goes up to 100% at 27 min, keeps until 30 min for the purpose of washing column, and then goes back to 35% for equilibrium. Data was collected at wavelength of 214 nm, 254 nm, and 326 nm. The injection volume was set at 5  $\mu$ L, and the flow rate was 1 mL/min.

The concentration of each PMF was obtained by calculating from the correlated standard curve. Data were expressed as mean  $\pm$  standard error mean (SEM). Statistical significance of mean difference between two groups was determined by student t-test. A significance level of  $p < 0.05$  was applied.

## **2.3 Results and Discussion**

### **2.3.1 HPLC Results**

High performance liquid chromatography (HPLC) was used to separate and identify each PMF in the Chenpi extracts. Standards were pre-tested on HPLC to determine the retention time as well as to establish the standard curve. The HPLC chromatogram was shown in Figure 2.1 and Figure 2.2.

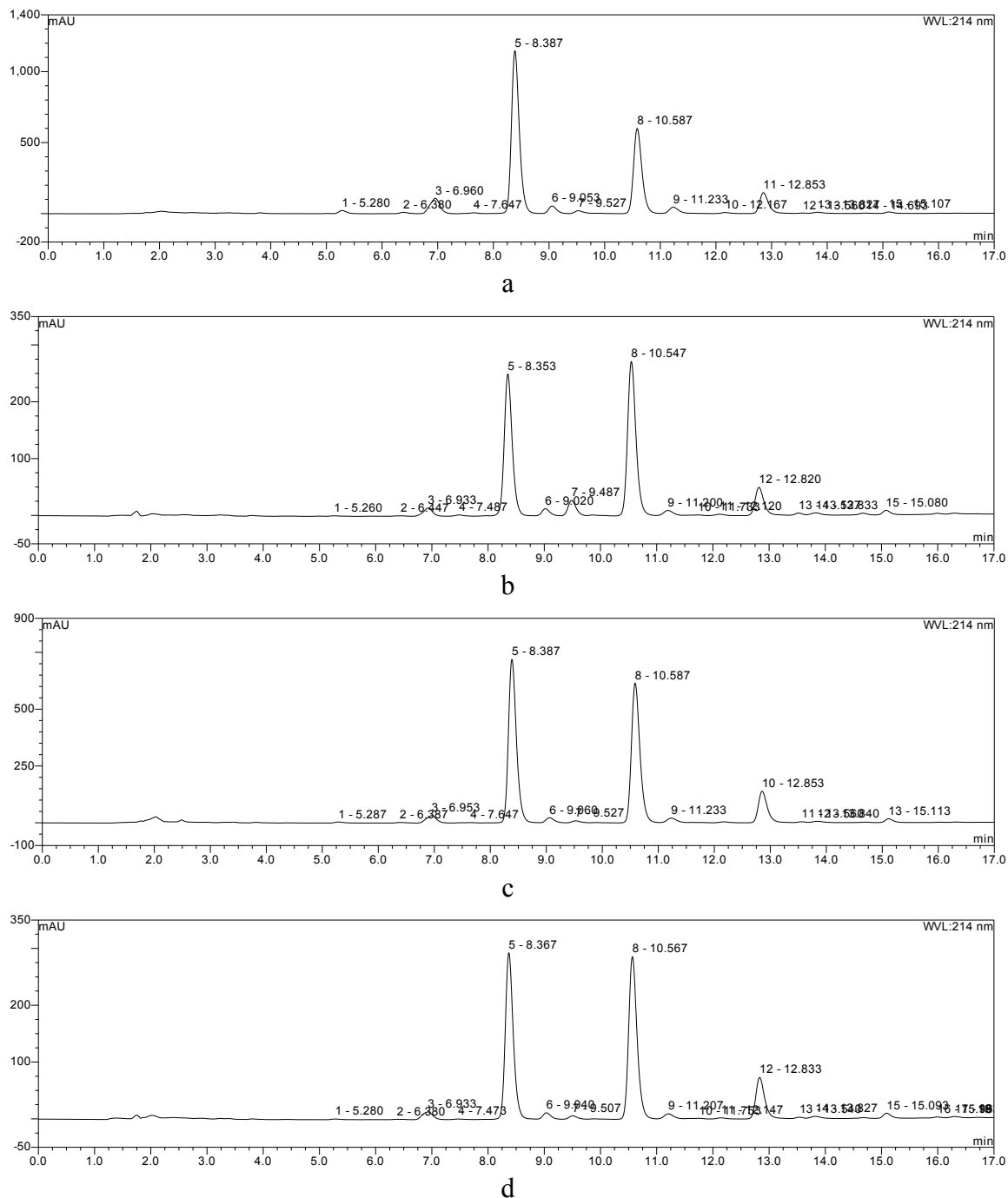


Figure 2.1 HPLC chromatograms of Polymethoxyflavones.

\*a. Jiangxi Chenpi extracted by Super-CFE (sample 1); b. Jiangxi Chenpi extracted by Sub-CFE (sample 2); c. Guangdong Chenpi extracted by Super-CFE (sample 3); d. Guangdong Chenpi extracted by Sub-CFE.

\*\*Peak 3: SIN; Peak 5: NOB; Peak 6: TetraMF; Peak 7: HeptaMF; Peak 8: TAN

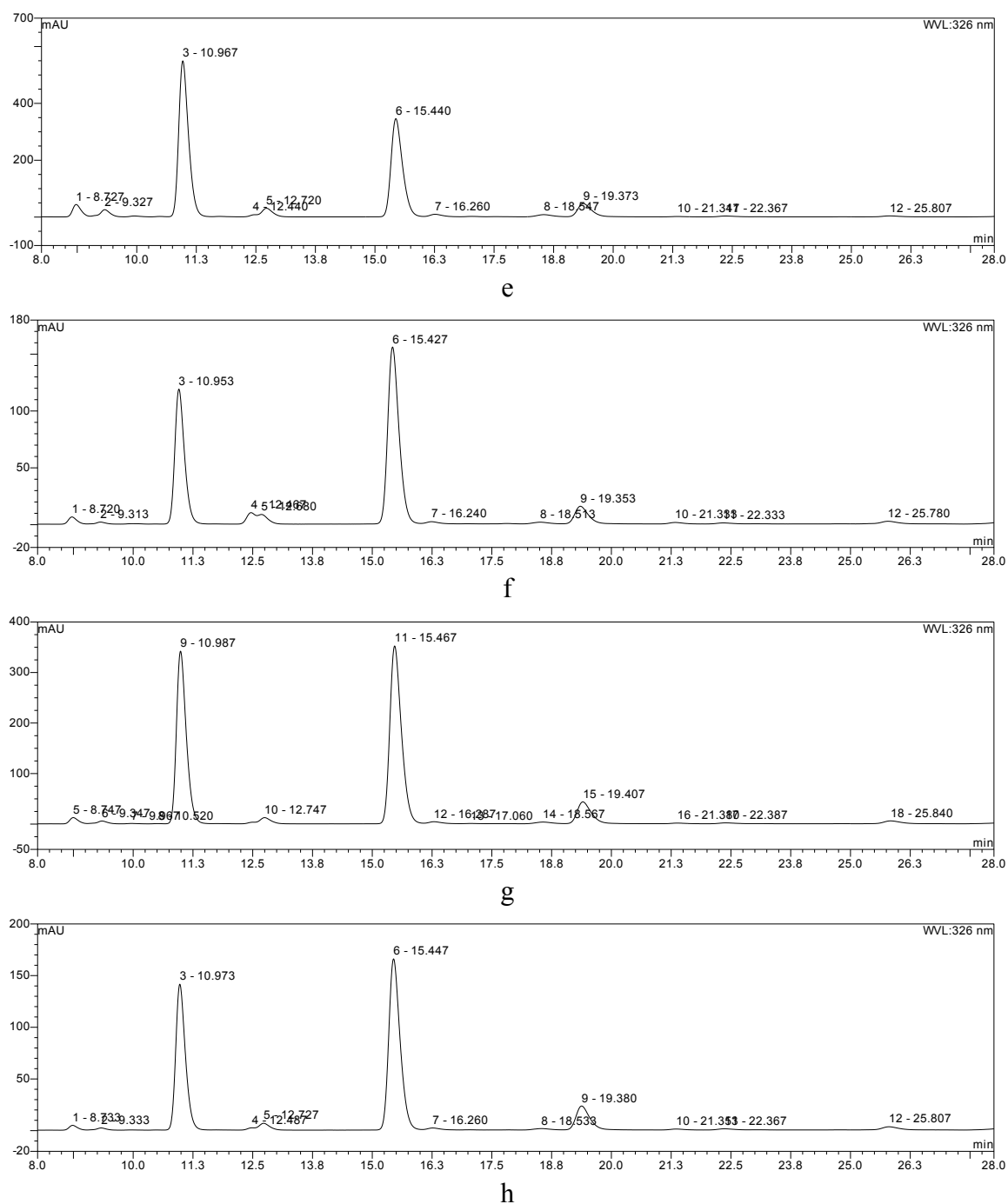


Figure 2.2 HPLC chromatograms of 5-Hydroxy-Polymethoxyflavones.

\*e. Jiangxi Chenpi extracted by Super-CFE (sample 1); f. Jiangxi Chenpi extracted by Sub-CFE (sample 2); g. Guangdong Chenpi extracted by Super-CFE (sample 3); h. Guangdong Chenpi extracted by Sub-CFE.

\*\*Peak 7: 5-OH-SIN; Peak 8: 5-OH-Hexa; Peak 9: 5-OH-NOB; Peak 10: 5-OH-HeptaMF; Peak 11: 5-OH-TetraMF; Peak 12: 5-OH-TAN

\*\*\*Peak 3: NOB; Peak 6: TAN

### 2.3.2 Concentration of PMFs in Chenpi Extracts

The concentration of each PMF was calculated from its standard curve based on the peak area from HPLC analysis. The data are presented in the form of *mg PMF per g extract*. Detailed data are recorded in Table 2.1.

According to the data shown in Table 2.1, basically, Chenpi extracted by Super-CFE had a much higher yield of PMFs than extracted by Sub-CFE: Jiangxi Chenpi extracted by Super-CFE had a yield of 843.90 mg PMFs/g extract, while only 264.13 mg PMFs/g extract when extracted by Sub-CFE; and Guangdong Chenpi extracted by Super-CFE gave 649.05 mg PMFs/g extract, however, only 392.26 mg PMFs/g extract when extracted by Sub-CFE.

The profiles of the four extracts were actually very similar. NOB and TAN, which accounted for more than 78% of the total PMFs, were definitely the two most abundant and common PMFs in Chenpi extracts, followed by SIN and HeptaMF. And this well explained that 5-OH-NOB and 5-OH-TAN became the two most abundant hydroxylated PMFs in Chenpi. Meanwhile, in any of the samples, HexaMF, 5-OH-TetraMF, and 5-OH-HeptaMF were not detected or detected in a concentration that is below standard curve reliable range, which is reasonable since their PMF counterparts are originally present in lower concentration. Furthermore, none of these extracts had the polar flavonoids, like hesperidin, which is owing to the non-polar fluids used in the extraction process.

By comparing the two extraction methods, it was found that it seemed like Super-CFE method tended to give a relatively higher level of NOB, while Sub-CFE would boost the yield of TAN; and similar phenomenon could be found on 5-OH-NOB and 5-OH-TAN. Apparently, different extraction methods would have different selectivity.

Table 2.1 Concentration of PMFs and 5-OH-PMFs in the four Chenpi extracts. Each data represents mean  $\pm$  SEM (n=3).

Concentration (mg/g extract)	Jiangxi Chenpi (Super-CFE)	Jiangxi Chenpi (Sub-CFE)	Guangdong Chenpi (Super-CFE)	Guangdong Chenpi (Sub-CFE)
SIN	37.46 $\pm$ 0.04	5.07 $\pm$ 0.02	11.75 $\pm$ 0.05	5.33 $\pm$ 0.02
HexaMF	N/A	N/A	N/A	N/A
NOB	411.27 $\pm$ 0.87	86.35 $\pm$ 0.39	260.95 $\pm$ 1.16	102.37 $\pm$ 0.40
TetraMF	3.77 $\pm$ 0.29	N/A	N/A	N/A
HeptaMF	8.21 $\pm$ 0.88	9.49 $\pm$ 0.04	2.64 $\pm$ 0.02	0.84 $\pm$ 0.14
TAN	262.13 $\pm$ 0.28	121.47 $\pm$ 0.50	271.17 $\pm$ 1.37	128.05 $\pm$ 0.49
5-OH-SIN	14.21 $\pm$ 0.05	7.50 $\pm$ 0.03	9.87 $\pm$ 0.02	7.51 $\pm$ 0.02
5-OH-HexaMF	18.12 $\pm$ 0.16	N/A	5.75 $\pm$ 0.08	N/A
5-OH-NOB	75.58 $\pm$ 0.67	22.80 $\pm$ 0.07	69.88 $\pm$ 0.23	35.60 $\pm$ 0.24
5-OH-TetraMF	N/A	N/A	N/A	N/A
5-OH-HeptaMF	N/A	N/A	N/A	N/A
5-OH-TAN	13.15 $\pm$ 0.05	11.46 $\pm$ 0.07	17.05 $\pm$ 0.39	12.56 $\pm$ 0.04
Total PMFs	722.84 $\pm$ 1.25	222.38 $\pm$ 0.95	546.50 $\pm$ 2.59	236.58 $\pm$ 1.01
Total 5-OH-PMFs	121.06 $\pm$ 0.85	41.75 $\pm$ 0.14	102.55 $\pm$ 0.35	55.68 $\pm$ 0.30
Total	843.90 $\pm$ 1.39	264.13 $\pm$ 1.08	649.05 $\pm$ 2.41	292.26 $\pm$ 1.19

### 2.3.3 Systemic Comparison of Jiangxi and Guangdong Chenpi

Due to the variation of each time extraction, data presented in the form of *mg PMF per g extract* was not sufficient to compare different sources. Therefore, another set of data presented as the percentage in total PMFs was prepared in Table 2.2. In this way, it was more obvious to see the composition of PMFs in each extract.

Table 2.2 Individual PMF percentages in total PMFs in the four Chenpi extracts. Each data represents mean  $\pm$  SEM (n=3). \*p < 0.05 indicates total PMFs of Jiangxi Chenpi extracted by Super-CFE is significantly greater than Guangdong Chenpi. \*\* p < 0.05 indicates total PMFs of Jiangxi Chenpi extracted by Sub-CFE is significantly greater than Guangdong Chenpi. \*\*\* p < 0.05 indicates total 5-OH-PMFs of Jiangxi Chenpi extracted by Super-CFE is significantly less than Guangdong Chenpi. \*\*\*\* p < 0.05 indicates total 5-OH-PMFs of Jiangxi Chenpi extracted by Sub-CFE is significantly less than Guangdong Chenpi.

% in Total PMFs	Jiangxi Chenpi (Super-CFE)	Jiangxi Chenpi (Sub-CFE)	Guangdong Chenpi (Super-CFE)	Guangdong Chenpi (Sub-CFE)
SIN	4.44 $\pm$ 0.01	1.92 $\pm$ 0.01	1.81 $\pm$ 0.01	1.82 $\pm$ 0.01
HexaMF	N/A	N/A	N/A	N/A
NOB	48.73 $\pm$ 0.10	32.69 $\pm$ 0.15	40.20 $\pm$ 0.18	35.03 $\pm$ 0.14
TetraMF	0.45 $\pm$ 0.03	N/A	N/A	N/A
HeptaMF	0.97 $\pm$ 0.10	3.59 $\pm$ 0.02	0.41 $\pm$ 0.003	0.29 $\pm$ 0.05
TAN	31.06 $\pm$ 0.03	45.99 $\pm$ 0.19	41.78 $\pm$ 0.21	43.81 $\pm$ 0.17
5-OH-SIN	1.68 $\pm$ 0.01	2.84 $\pm$ 0.01	1.52 $\pm$ 0.003	2.57 $\pm$ 0.01
5-OH-HexaMF	2.15 $\pm$ 0.02	N/A	0.89 $\pm$ 0.01	N/A
5-OH-NOB	8.96 $\pm$ 0.08	8.63 $\pm$ 0.03	10.77 $\pm$ 0.03	12.18 $\pm$ 0.08
5-OH-TetraMF	N/A	N/A	N/A	N/A
5-OH-HeptaMF	N/A	N/A	N/A	N/A
5-OH-TAN	1.56 $\pm$ 0.01	2.63 $\pm$ 0.03	2.63 $\pm$ 0.06	4.30 $\pm$ 0.01
Total PMFs	85.65 $\pm$ 0.15 *	84.20 $\pm$ 0.36 **	84.20 $\pm$ 0.40 *	80.95 $\pm$ 0.35 **
Total 5-OH-PMFs	14.35 $\pm$ 0.10 ***	15.80 $\pm$ 0.05 ****	15.80 $\pm$ 0.05 ***	19.05 $\pm$ 0.10 ****
Total	100	100	100	100

By comparing Jiangxi Chenpi and Guangdong Chenpi, no matter extracted by which method, the trend is Jiangxi Chenpi had more PMF content, while Guangdong Chenpi had more 5-OH-PMF content. Jiangxi Chenpi extracted by Super-CFE had 85.65% PMFs and 14.35% 5-OH-PMFs, and it had 84.2% PMFs and 15.8% 5-OH-PMFs when extracted by Sub-CFE; meanwhile, Guangdong Chenpi extracted by Super-CFE had 84.2% PMFs and 15.8% 5-OH-PMFs, and, if extracted by Sub-CFE, it had 80.95% PMFs and 19.05% 5-OH-PMFs. Significant difference had been proved by student t-test.

In China, Guangdong Chenpi is famous for having the best quality and medical treatment effects, which renders it the most expensive Chenpi in the market. It is reported that some hydroxylated PMFs have better biological functions over PMFs, such as anti-cancer and anti-inflammatory effects (Li et al., 2009). Since Guangdong Chenpi has a relative higher level of 5-OH-PMFs, it is not hard to imagine its potent biological functions. In addition, the years of storage can also elevate the value of Chenpi for the similar reason, for which more hydroxylated PMFs are produced by bacterial activities during the storage.

## **2.4 Conclusion**

To conclude, both of the Super-CFE and Sub-CFE method have the advantages over traditional extraction methods. They are time saving and labor saving, and give better selectivity and yield of production. Super-CFE is definitely the most promising method among the extraction methods. As the market of functional food grows, these methods will play important roles in pulling out health-beneficial compounds from natural sources.

Chenpi has been used in Asian countries as a traditional medicine for thousands of years, and its secret has been discovered gradually. A detailed database of Chenpi profile is necessary in order to perform systemic comparison.



## **Chapter 3 Separation and Purification of Polymethoxyflavones by Column Chromatography**

### **3.1 Principle**

In recent years, functional food and nutraceutical industry has become a popular field since more and more people put health and well-being to the first place. Flavonoids from plant sources have been proved having numerous health benefits, such as anti-inflammation, antioxidant, anti-cancer, and so forth. Polymethoxyflavones (PMFs) are a group of unique flavonoids found mainly in citrus peels. Up to date, there are more than 20 polymethoxylated flavonoids have been isolated and identified from different parts of citrus plants (Li, Lo, Dushenkov, & Ho, 2008). Due to the potent biological activities of PMFs, there is a demand of obtaining purified compounds. Thus, a rapid and cost-effective separating method is always needed to purify large quantities of PMFs from citrus peels.

As technology develops, more and more fancy techniques have been established to separate and isolate natural compounds. These techniques include preparative HPLC system (Wang, Wang, Huang, Tu, & Ni, 2007), automatic flash chromatography (Uckoo, Jayaprakasha, & Patil, 2011), supercritical fluid chromatography (Li, Lambros, Wang, Goodnow, & Ho, 2007), high-speed counter-current chromatography (Wang et al., 2005), and so on. Unfortunately, these methods are mainly used for small-scale separation. When speaking of large quantities, column chromatography is always the first choice even though it has many drawbacks, including requesting for time, labor, and huge

amount of solvents. The biggest advantage of column chromatography is that it has a broad range of separation from milligrams to grams, or even larger. In this study, silica gel column chromatography was used to separate PMFs from aged orange peel extracts.

## **3.2 Experimental**

### **3.2.1 Materials**

Aged orange peel extracts were provided by Dr. Qingrong Huang (Food Science Department, Rutgers, the State University of New Jersey, USA). These samples were prepared in the South China Agricultural University using two extraction methods: super-critical fluid extraction (Super-CFE) and sub-critical fluid extraction (Sub-CFE).

Acetonitrile (HPLC grade), water (HPLC grade), methanol (HPLC grade), ethyl acetate (ACS grade), and hexanes (ACS grade), dichloromethane (ACS grade) were purchased from Pharmco-AAPER (Brookfield, CT, USA). Silica gel (60 Å, 40-63 µm, standard grade) was purchased from Sorbent Technologies (Norcross, GA, USA).

### **3.2.2 Apparatus**

The glass column (24/40 outer joint, 1 in ID x 12 in E.L., 2 mm Stpk) from Chemglass Life Sciences (Vineland, NJ, USA) was used to prepare the silica gel column.

The HPLC system consisted of a Dionex UltiMate 3000 HPLC series (Sunnyvale, CA, USA) including an UltiMate 3000 Pump, an UltiMate 3000 Variable Wavelength Detector, and an UltiMate 3000 Auto-sampler. Chromeleon software was used to perform instrument control and data analysis. Supelco Ascentis® RP-Amide C18 HPLC column (15 cm X 4.6 mm, 3  $\mu$ m) was purchased from Sigma-Aldrich (St. Louis, MO, USA).

### 3.2.3 Method

The method started from mixing 45 g silica gel with hexanes until it became a slurry-type suspension. This silica gel suspension, as the stationary phase, was carefully poured into the glass column (24/40 outer joint, 1 in ID x 12 in E.L., 2 mm Stpk). Pressure is applied to pack the column into a denser manner through connecting a gas tank to the top of the column. A thin layer of sea sand was then added on the top of silica gel to prevent any possible disruption of stationary phase from adding mobile phase.

1 g orange peel extract from super-critical extraction method was firstly dissolved in 3 mL dichloromethane, and then loaded from the top of sea sand layer. After the orange peel sample was fully absorbed by the silica gel, 200 mL hexanes was added to the start the elution.

The elution system consisted of hexanes, ethyl acetate, and methanol, starting from hexanes/ethyl acetate (V/V) = 100/0 to hexanes/ethyl acetate (V/V) = 0/100 with 10% change each time, and finally switching to ethyl acetate/methanol (V/V) = 95/5.

Silica gel has strong affinity with higher-polarity compounds. As increasing the percentage of ethyl acetate, the polarity of the mobile phase is elevated; thus, compounds with high polarity come out last. Due to different affinities with silica gel, targeted compounds come out at different solvent gradient. Generally, 0.1% acetic acid was added in the mobile phase to help compounds coming out fast and complete each time.

One drop of elute was placed on the thin layer chromatography (TLC) plate, and the appearance and disappearance of a targeted compound was determined by the signal via UV lamp. Once the signal was gone, the mobile phase was changed to the next gradient. All the fractions were kept separately, and sent to HPLC analysis using the following program:

The mobile phase consists of water (A) and acetonitrile (C); and the 20 min gradient starts with 40% C, then linearly increases to 55% C in 10 min, and then linearly increases to 70% C in 15 min, finally stays at 80% C at 20 min; then increases to 100% C in 21 min and keeps there for 4 min to wash the column, and then goes back to 40% C for equilibrium. Data was collected at wavelength of 214 nm, 254 nm, and 326 nm. The injection volume was set at 5  $\mu$ L, and the flow rate was 1 mL/min.

After the compound was confirmed, the solvents were evaporated and then freeze-dried. Further identification was carried out by LC-MS and NMR.

### 3.3 Results and Discussion

#### 3.3.1 Detailed conditions of column chromatography

The aged orange peel extracts used in this study have three major PMFs, namely nobiletin (NOB), tangeretin (TAN), and 5-demethyl-nobiletin (5-OH-NOB) (Figure 3.1). Other PMFs are present in trace concentrations. Therefore, the purpose of this study was to obtain relative pure NOB, TAN, and 5-OH-NOB using silica gel column chromatography.

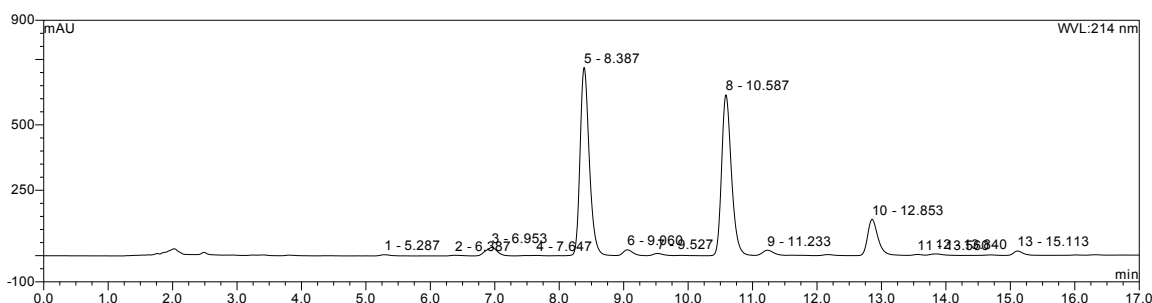


Figure 3.1 HPLC chromatogram of aged orange peel sample.

\* Peak 5: NOB; Peak 8: TAN; Peak 10: 5-OH-NOB

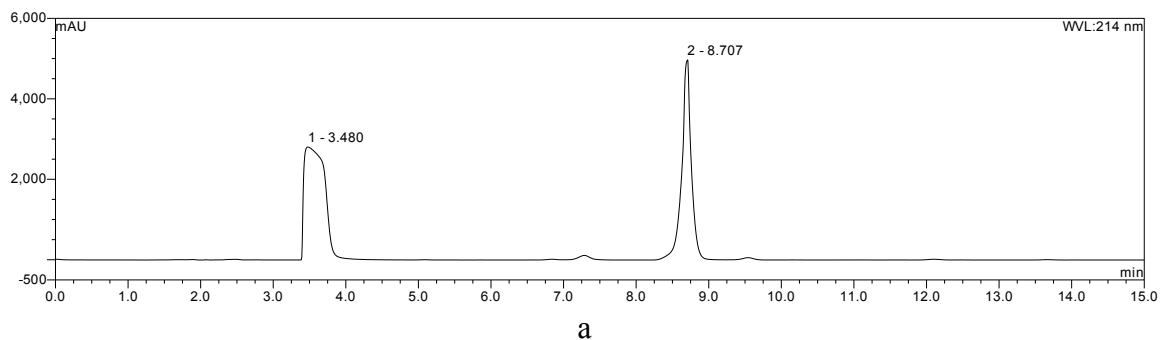
Mobile phase at each gradient was evaluated. It was found that, at the gradient hexanes/ethyl acetate (V/V) = 60/40, 50/50, 40/60, and 30/70, targeted PMFs were flashed out and the fractions were saved. Detailed conditions are recorded in Table 3.1. Other gradients did not deliver interested compounds, however, they helped to remove the impurities. Fractions from other gradients were dumped into waste directly.

Table 3.1 Optimal conditions of silica gel column chromatography on separating PMFs.

Elution Gradient (Hexanes/Ethyl Acetate = V/V)	Volume	Targeted PMFs
60/40	600	5-OH-NOB
50/50	800	TAN
40/60	800	NOB
30/70	600	SIN

### 3.3.2 HPLC results

HPLC analysis was used primarily to detect the compounds from the silica gel column chromatography and confirm their purity. Figure 3.2 shows the three targeted PMFs separated from the silica gel column, namely NOB, TAN, and 5-OH-NOB, with a purity of 97, 98, and 96%, respectively. The retention time was 8.7 min for NOB, 10.9 min for TAN, 13.6 min for 5-OH-NOB, and 3.4 min for ethyl acetate. The HPLC results indicate that silica gel column chromatography can separate these three PMFs to an excellent purity.



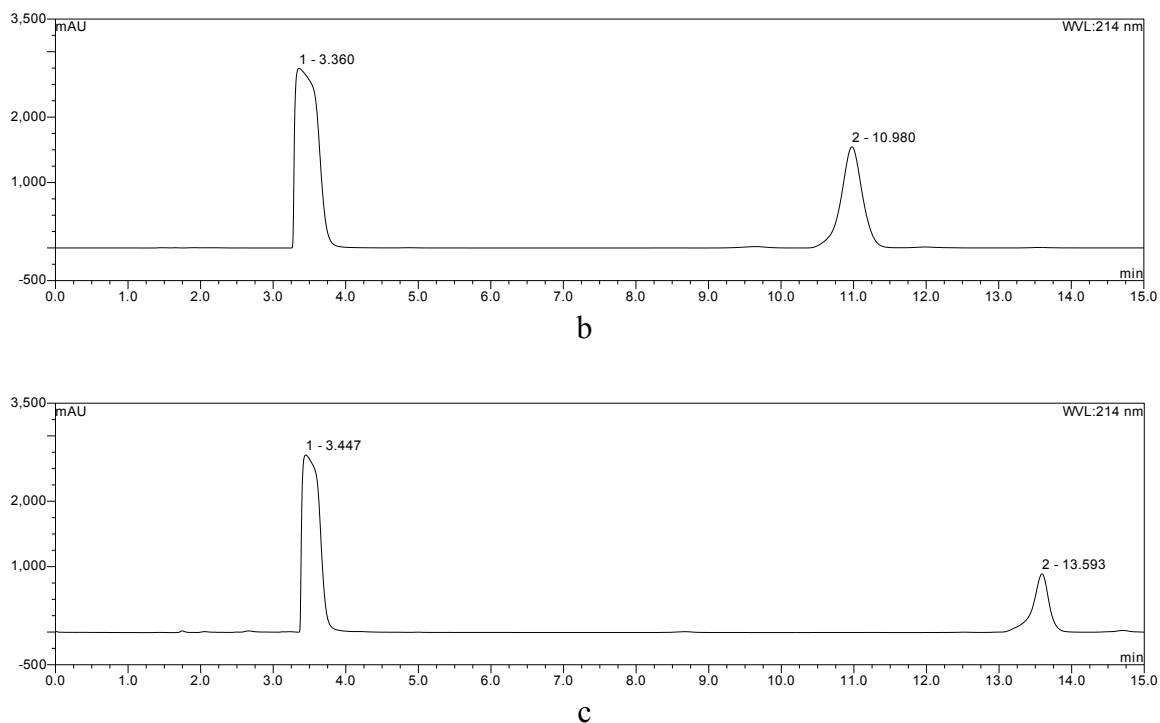


Figure 3.2 HPLC chromatograms of purified NOB (a), TAN (b), and 5-OH-NOB (c).

\*Peak 1 is ethyl acetate.

\*\*Peak 2: NOB (a), TAN (b), and 5-OH-NOB (c).

### 3.4 Conclusion

In conclusion, silica gel column chromatography is a good method to achieve the following purposes: 1) to separate individual PMFs with good purity from a mixture or a natural orange peel extract; 2) to isolate both small and large quantities of PMFs by adjusting the column size and amount of silica gel added; 3) to purify an orange peel extract by removing oils and waxes or the polar flavonoids.

Silica gel column chromatography is also utilized in other experiments where purification of a certain PMF is needed, such as the experiment of demethylation and acetylation reaction, which will be covered in Chapter 4.

Since the PMFs recovered from silica gel column chromatography have high purities, they can be used for chemical modification or directly for in-vitro or in-vivo studies.



## **Chapter 4 Preparation of Demethylated and Acetylated Polymethoxyflavone Derivatives Through Chemical Syntheses**

### **4.1 Principle**

Polymethoxyflavones (PMFs), which are a unique group of flavonoids present almost exclusively in citrus peels, contain multiple methoxy groups on the C6-C3-C6 backbone. Hydroxylated polymethoxyflavones (OH-PMFs) are present in citrus peels as well but with much lower concentration.

Some OH-PMFs were previously reported having stronger bioactivities than their PMF counterparts (Li et al., 2009). Other studies also found that hydroxyl groups play important roles in bioactivities. For example, the hydroxyl groups of quercetin do a great job in inhibiting proliferation and inducing apoptosis (Sakao, Fujii, & Hou, 2009). The hydroxyl groups on the B-ring of flavonoids would promote the antioxidant activity (Kongpichitchoke, Hsu, & Huang, 2015). Therefore, the study on OH-PMFs will be of great interest. The conversion from PMF to 5-OH-PMF is a one-step synthesis in acidic condition (Figure 4.1).

In pharmaceutical industry, *pro-drug* is a crucial term, which explains a medication that is converted from inactive form to active form through a metabolic process in the body. Basically, pro-drug is used to deliver the active compound, and prevent it from undesired reactions or improve its bioavailability. Epigallocatechin-3-gallate (EGCG) had an improved bioavailability when presented in the form of acetylated EGCG; and it was

converted back to EGCG both in vitro and in vivo (Lambert et al., 2006). This tells us that acetylated PMFs could potentially increase the bioavailability of hydroxylated PMFs.

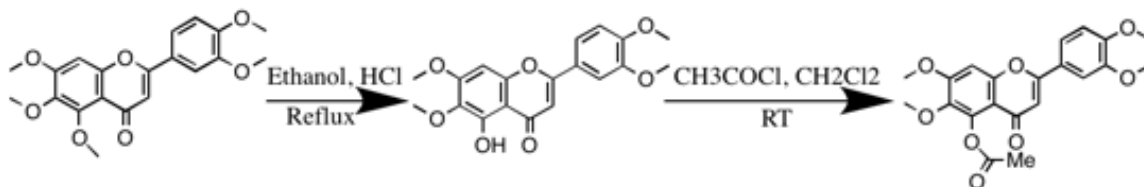


Figure 4.1 Chemical demethylation and acetylation of PMFs.

In this study, we chemically converted nobiletin (NOB) and sinensetin (SIN) to their 5-hydroxyl and 5-acetyl derivatives, and converted 5-hydroxy-3,6,7,8,3',4'-hexamethoxyflavone (5-OH-HeptaMF) to its 5-acetyl derivative. Selected PMFs and their derivatives were used in the final permeability experiment.

## 4.2 Experimental

### 4.2.1 Materials

SIN and 5-OH-HeptaMF were kindly provided by Dr. Shiming Li (Food Science Department, Rutgers, the State University of New Jersey, USA). NOB was separated from aged orange peel extracts, which was discussed in Chapter 3.

Ethyl alcohol (200 proof-absolute, anhydrous, ACS grade), anhydrous dichloromethane (ACS grade), Acetonitrile (HPLC grade), water (HPLC grade), methanol (HPLC grade),

ethyl acetate (ACS grade), and hexanes (ACS grade) were purchased from Pharmco-AAPER (Brookfield, CT, USA). Acetyl chloride (>98.5% ACS grade) and hydrochloric acid (37%) were purchased from Fisher Scientific (Fairlawn, NJ, USA). Anhydrous pyridine was purchased from Sigma-Aldrich (St. Louis, MO, USA). Silica gel (60 Å, 40-63 µm, standard grade) was purchased from Sorbent Technologies (Norcross, GA, USA).

#### **4.2.2 Apparatus**

The glass column (24/40 outer joint, 1 in ID x 12 in E.L., 2 mm Stpk) from Chemglass Life Sciences (Vineland, NJ, USA) was used to prepare the silica gel column.

The HPLC system consisted of a Dionex UltiMate 3000 HPLC series (Sunnyvale, CA, USA) including an UltiMate 3000 Pump, an UltiMate 3000 Variable Wavelength Detector, and an UltiMate 3000 Auto-sampler. Chromeleon software was used to perform instrument control and data analysis. Supelco Ascentis® RP-Amide C18 HPLC column (15 cm X 4.6 mm, 3 µm) was purchased from Sigma-Aldrich (St. Louis, MO, USA).

#### **4.2.3 Method**

##### **1). Demethylation reaction**

About 500 mg NOB was dissolved into 150 mL Ethyl alcohol (200 proof-absolute, anhydrous, ACS grade), and 20 mL hydrochloric acid (37%) was gradually dropped into

the solution. This solution was heated and refluxed for about 24 hours. The reaction was monitored by HPLC analysis. Every time a drop of reaction mixture was dispersed into methanol, and then sent to HPLC using the following program:

The mobile phase consists of water (A) and acetonitrile (C); and the 20 min gradient starts with 40% C, then linearly increases to 55% C in 10 min, and then linearly increases to 70% C in 15 min, finally stays at 80% C at 20 min; then increases to 100% C in 21 min and keeps there for 4 min to wash the column, and then goes back to 40% C for equilibrium. Data was collected at wavelength of 214 nm, 254 nm, and 326 nm. The injection volume was set at 5  $\mu$ L, and the flow rate was 1 mL/min.

After the reaction was finished, the solvents were removed by Rotavapor. Then the residue was re-dissolved in 100 mL ethyl acetate/water (V/V) = 1/1. After removing the water layer, the ethyl acetate layer was washed with water three times. The combined water fraction was re-extracted by ethyl acetate to extract remaining PMFs. The ethyl acetate fraction was dried by anhydrous sodium sulfate first, and then the solvent was removed by Rotavapor.

## 2). Acetylation reaction

About 100 mg 5-OH-NOB was dissolved in 50 mL anhydrous dichloromethane, followed by the addition of 10 mL acetyl chloride. After stirring the mixture for 5 minutes, 3 mL anhydrous pyridine was gradually dropped into the solution. The resulting solution was

stirred under ambient temperature for about 24 hours. The reaction was monitored by HPLC analysis. Every time a drop of reaction mixture was dispersed into methanol, and then sent to HPLC using the same program as mentioned above.

After the reaction was finished, the mixture was concentrated by Rotavapor. Then the residue was re-dissolved in 100 mL ethyl acetate/water (V/V) = 1/1. After removing the water layer, the ethyl acetate layer was washed with water, 1 N hydrochloric acid, water, and brine, and further dried over anhydrous sodium sulfate. After filtered, the ethyl acetate was concentrated by Rotavapor.

### 3). Purification of PMF derivatives

Silica gel column chromatography was used to purify the generated products, including 5-OH-NOB, 5-OH-SIN, 5-Ac-NOB, 5-Ac-SIN, and 5-Ac-HeptaMF. Impurities contain the remaining substrates, impurities from the substrates, any by-products generated, and so on.

The concentrated reaction residue was dissolved 1 mL dichloromethane, and loaded onto silica gel column, which was the glass column (24/40 outer joint, 1in ID x 12 in E.L., 2 mm Stpk) packed with 45 g silica gel. The elution system consisted of hexanes and ethyl acetate. Detailed method was discussed in Chapter 3. Purified compounds get flashed out at a certain gradient of hexanes/ethyl acetate (V/V) (Table 4.1).

Table 4.1 Conditions of silica gel column chromatography on purifying PMF derivatives from chemical reactions.

PMF derivatives	Elution Gradient (Hexanes/Ethyl Acetate = V/V)
<b>5-OH-NOB</b>	<b>60/40</b>
<b>5-OH-SIN</b>	<b>50/50</b>
<b>5-Ac-NOB</b>	<b>50/50</b>
<b>5-Ac-SIN</b>	<b>40/60</b>
<b>5-Ac-HeptaMF</b>	<b>60/40</b>

Again, the purities of PMF derivatives were analyzed by HPLC. After the compound was confirmed, the fraction was evaporated and then freeze-dried. Further identification was carried out by LC-MS and NMR.

## 4.3 Results and Discussion

### 4.3.1 Yield of production

Overall speaking, the demethylation reaction and acetylation reaction generate high yield of products. 5-OH-PMFs are present in natural orange peel but with trace concentrations. 5-Ac-PMFs are not present in natural orange peel at all. With chemical synthesis, we are able to obtain large amount of 5-OH-PMFs and 5-Ac-PMFs, and further study on their properties.

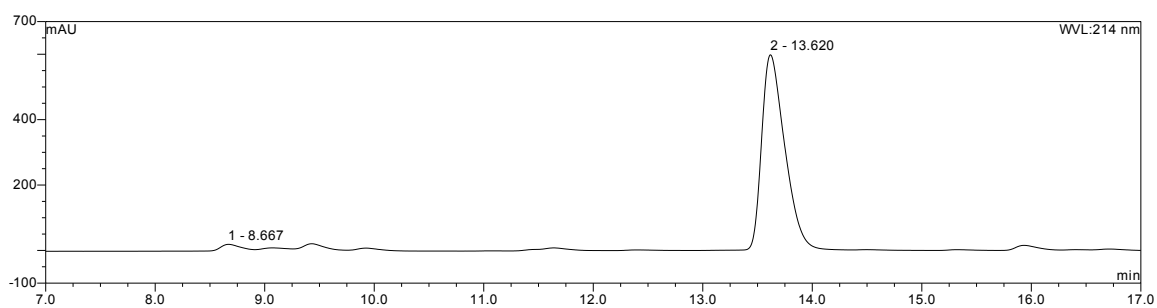
The production rate of each reaction was more than 60% (Table 4.2), and this was counted from the synthesized compound after purified and freeze dried. Note that this is different from the HPLC analysis, which is covered in the next section. There are some loss occurred during the post-reaction handling.

Table 4.2 Yield of PMF derivatives. \*The weight of synthesized derivative was measured after purified and freeze dried.

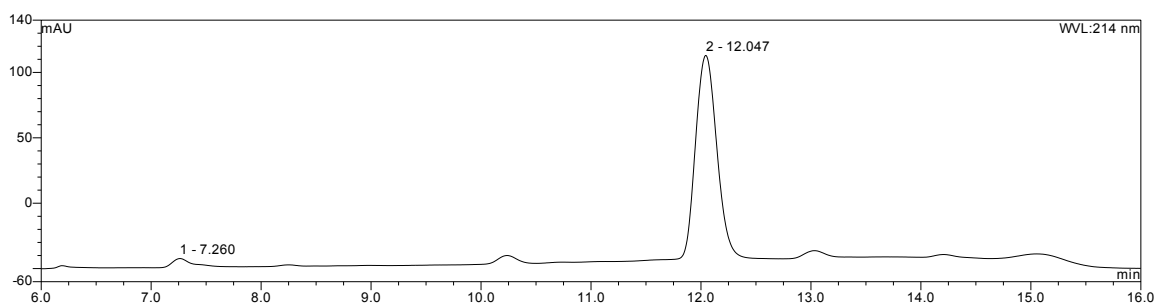
<b>PMFs or 5-OH-PMFs</b>	<b>Synthesized derivatives*</b>	<b>Production</b>
NOB (0.5864 g)	5-OH-NOB (0.3976 g)	<b>67.80%</b>
5-OH-NOB (0.2210 g)	5-Ac-NOB (0.1338 g)	<b>60.54%</b>
SIN (0.5911 g)	5-OH-SIN (0.4123 g)	<b>69.75%</b>
5-OH-SIN (0.1182 g)	5-Ac-SIN (0.0879 g)	<b>74.37%</b>
5-OH-HeptaMF (0.3000 g)	5-Ac-HeptaMF (0.2016 g)	<b>67.20%</b>

#### 4.3.2 HPLC results

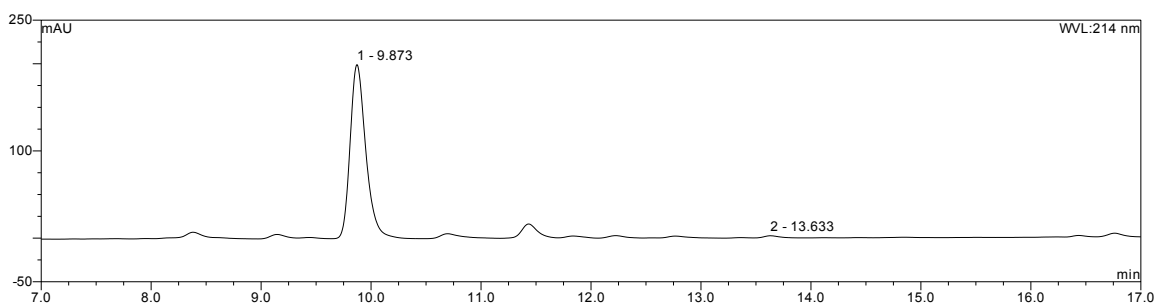
In this study, all the reactions were monitored by HPLC analysis to determine the end point of the reaction. By only looking at the peak areas of substrate and product on the HPLC chromatogram, we are able to determine how much the substrate was converted into product. The relative area percentages of 5-OH-NOB, 5-OH-SIN, 5-Ac-NOB, 5-Ac-SIN, and 5-OH-HeptaMF are 97.92, 96.00, 99.27, 88.79, and 91.85%, respectively (Figure 4.2), which indicates by the time almost all the substrates were converted and the reaction reached the end point. By applying silica gel column chromatography, the products were further purified, and the final purities of the PMF derivatives were all above 95%.



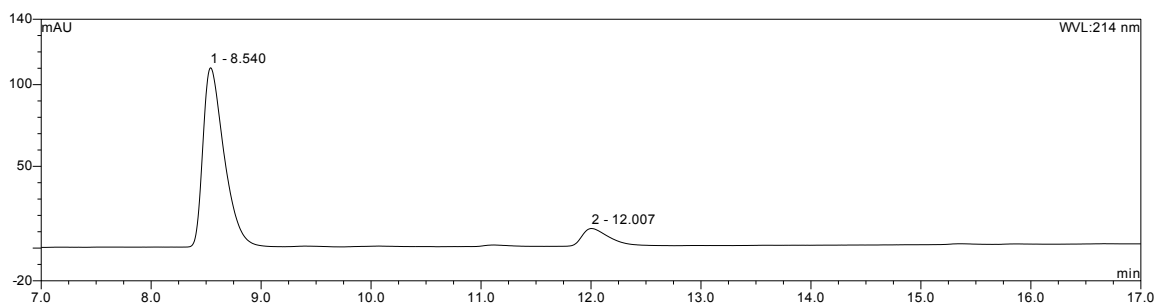
a



b



c



d



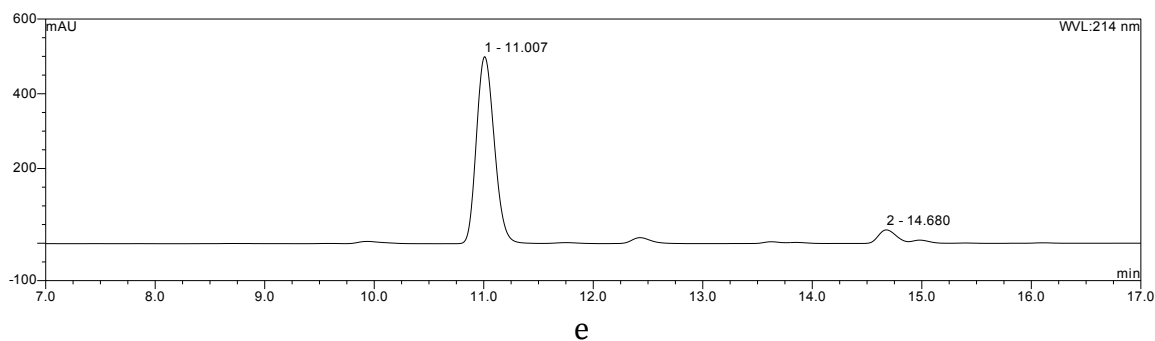


Figure 4.2 HPLC chromatograms of reaction mixture at end point.

- a. Demethylation on NOB (peak 1: NOB and peak 2: 5-OH-NOB)
- b. Demethylation on SIN (peak 1: SIN and peak 2: 5-OH-SIN)
- c. Acetylation on 5-OH-NOB (peak 1: 5-Ac-NOB and peak 2: 5-OH-NOB)
- d. Acetylation on 5-OH-SIN (peak 1: 5-Ac-SIN and peak 2: 5-OH-SIN)
- e. Acetylation on 5-OH-Hepta (peak 1: 5-Ac-HeptaMF and peak 2: 5-OH-HeptaMF)

#### 4.4 Conclusion

This study investigated the optimal condition of two chemical modifications on PMFs: demethylation and acetylation. The results revealed that the synthesis methods coupled with HPLC analysis are feasible and reliable to produce 5-OH-PMFs and 5-Ac-PMFs. The conversion rate reached as high as 90% during the 24 hours reaction. It was exciting to obtain high-purity PMF derivatives, and some of them were used in the permeability study.

## **Chapter 5 Solubility Test on Polymethoxyflavones and Their Derivatives**

### **5.1 Principle**

One of the important factors of absorption is solubility, which mainly refers to aqueous solubility across a pH range. The structure of Polymethoxyflavones (PMFs), which has multiple methoxy groups, clearly indicates their poor aqueous solubility. The lyophilisation solubility assay (LYSA) conducted by Li, Wang, Ho, and Dushenkov (2008) revealed that the overall solubility of PMFs is poor, but the more hydroxyl groups the PMF has, the better soluble it would be.

In the concept of bioavailability, solubility is not an independent parameter, which means it works with other parameters like permeability. The Lipinski's rule of five marks the criteria of an orally active drug for its pharmacokinetics in human body, including absorption, distribution, metabolism, and excretion (ADME). The rules are as followings:

There are no more than 5 H-bond donors

There are no more than 10 H-bond acceptors

The molecular weight is less than 500

The octanol-water partition coefficient (Log P) is less than 5

(Lipinski, Lombardo, Dominy, & Feeney, 2012)

For the PMFs used in the study, they all have less than 5 H-bond donors, less than 10 H-bond acceptors, and less than 500 molecular weight. There is no previous paper recording the octanol-water partition coefficient, thus, we performed related experiments on selected PMFs.

The so-called shake-flask method was utilized in this solubility study. Basically, the substrate is added into two immiscible liquids in a test tube – usually octanol/water system – and then measure the concentration of the substrate in the two liquids separately by spectroscopic methods, like HPLC, GC, and so on (Berthod & Carda-Broch, 2004). In this study, we applied *shake-flask* method, and used HPLC to determine the distributions of PMFs and their derivatives in octanol and water.

## 5.2 Experimental

### 5.2.1 Materials

Sinensetin (SIN), 3,5,6,7,8,3',4'-Heptamethoxyflavone (HeptaMF), and 5-hydroxy-3,6,7,8,3',4'-hexamethoxyflavone (5-OH-HeptaMF) were kindly provided by Dr. Shiming Li (Food Science Department, Rutgers, the State University of New Jersey, USA). 5-demethyl-sinensetin (5-OH-SIN), 5-acetyl-sinensetin (5-Ac-SIN), and 5-acetyl-3,6,7,8,3',4'-hexamethoxyflavone (5-Ac-HeptaMF) were previously prepared as mentioned in Chapter 4.

Acetonitrile (HPLC grade), water (HPLC grade), and methanol (HPLC grade) were purchased from Pharmco-AAPER (Brookfield, CT, USA). 1-octanol (99%) and Dimethyl sulfoxide (>99.9%, ACS grade) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

### **5.2.2 Apparatus**

The HPLC system consisted of a Dionex UltiMate 3000 HPLC series (Sunnyvale, CA, USA) including an UltiMate 3000 Pump, an UltiMate 3000 Variable Wavelength Detector, and an UltiMate 3000 Auto-sampler. Chromeleon software was used to perform instrument control and data analysis. Supelco Ascentis® RP-Amide C18 HPLC column (15cm X 4.6mm, 3µm) was purchased from Sigma-Aldrich (St. Louis, MO, USA).

### **5.2.3 Method**

SIN, 5-OH-SIN, 5-Ac-SIN, HeptaMF, 5-OH-HeptaMF, and 5-Ac-HeptaMF, respectively, were prepared into 10 mg/ml solutions in DMSO. Then the solutions were diluted 100 times into a mixture of water/octanol (V/V = 1:1). The obtained mixtures were vortexed, and then left until equilibrated. Both the water and octanol layers were analyzed by HPLC at the following program:

The mobile phase consists of water (A) and acetonitrile (C); and the 20 min gradient starts with 40% C, then linearly increases to 55% C in 10 min, and then linearly increases

to 70% C in 15 min, finally stays at 80% C at 20 min; then increases to 100% C in 21 min and keeps there for 4 min to wash the column, and then goes back to 40% C for equilibrium. Data was collected at wavelength of 214 nm, 254 nm, and 326 nm. The injection volume was set at 5  $\mu$ l, and the flow rate was 1 ml/min.

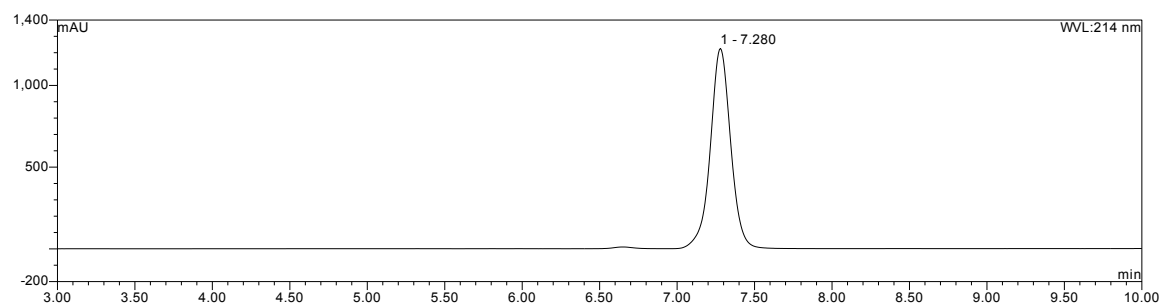
Peak areas of the interested compounds were obtained from HPLC, and the concentrations were calculated from standard curves. Data were expressed as mean  $\pm$  standard error mean (SEM).

### **5.3 Results and Discussion**

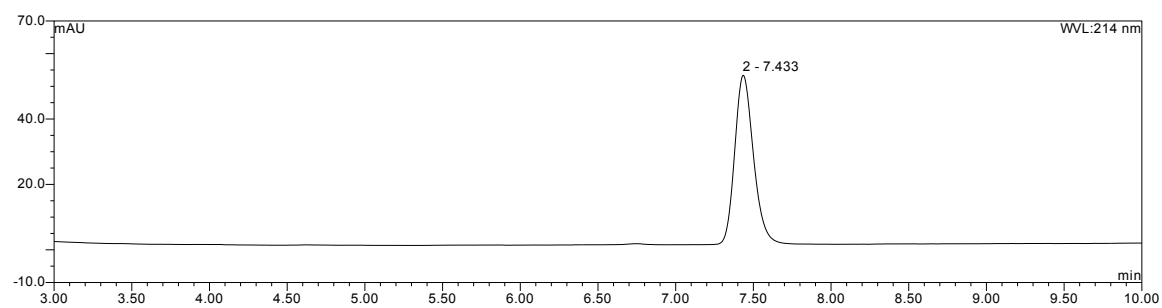
#### **5.3.1 HPLC results**

The HPLC results tell that PMFs and their derivatives generally have very low aqueous solubility, meaning nearly all the compounds distributed in the octanol layer. SIN had a peak area of 186.68 in octanol layer, but 7.27 in water layer, both detected at 214 nm wavelength (Figure 5.1). This indicates SIN was 26 times more distributed in octanol than in water. HeptaMF has an even worse water solubility, for which the peak area is 136.96 in octanol, but only 1.41 in water (Figure 5.2), meaning HeptaMF was 97 times more distributed in octanol than in water. This result is actually quite reasonable because HeptaMF contains two more methoxy groups than SIN. As for 5-Ac-SIN and 5-Ac-HeptaMF, they are less aqueous-soluble than their counterpart PMFs. 5-Ac-SIN

distributed over 70 times in octanol than in water, and 5-Ac-HeptaMF distributed over 121 times in octanol than in water.

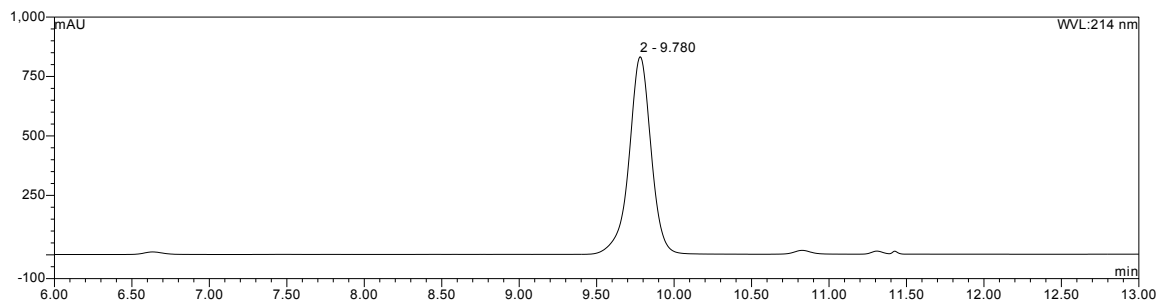


a

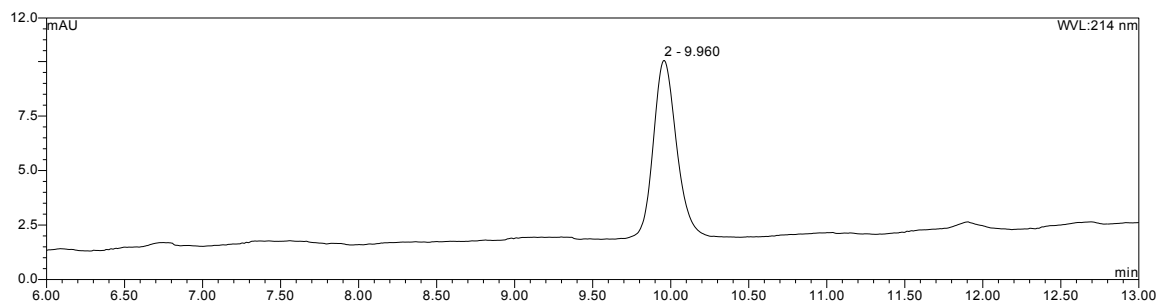


b

Figure 5.1 HPLC chromatogram of SIN in octanol (a) and water (b).



c



d

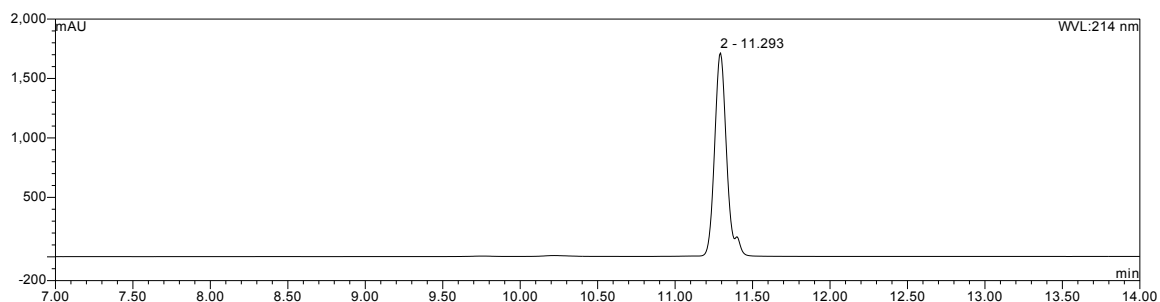
Figure 5.2 HPLC chromatogram of HeptaMF in octanol (c) and water (d).

There were barely 5-OH-SIN or 5-OH-HeptaMF distributed in water, which reveals 5-OH-PMFs probably have the poorest aqueous solubility. One interesting observation on 5-OH-PMFs is that, with the presence of octanol, the HPLC retention time was changed. 5-OH-PMFs are expected to be more polar than PMFs due to the presence of hydroxyl group. However, the elution orders on normal phase silica gel column and C<sub>18</sub> reverse phase HPLC suggest that 5-OH-PMFs would be more non-polar. For example, from the silica gel column, 5-OH-NOB is eluted earlier than NOB; on the contrary, 5-OH-NOB comes out later than NOB on the reverse phase HPLC system. This is due to the formation of stable six-member ring from the intramolecular hydrogen bond between the

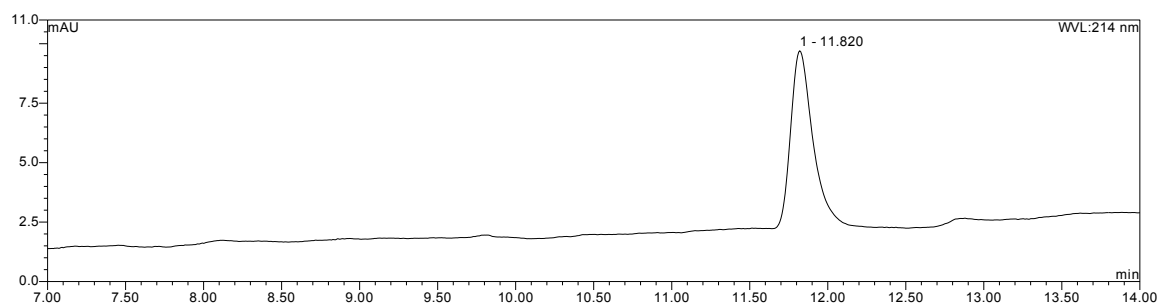
hydrogen on 5-hydroxyl group and the oxygen on the 4-carbonyl group (Li, Lo, & Ho, 2006). Octanol has a unique property, which is hydrophobic on the body but hydrophilic at the tail. When octanol is introduced to 5-OH-PMF, the hydrophobic body will bind to PMF, while the hydrophilic tail tends to bind to the oxygen on the 4-carbonyl group. This results in the breakage of the hydrogen bond between the hydrogen on 5-hydroxyl group and the oxygen on the 4-carbonyl group. Thus, 5-OH-PMFs dissolved in octanol have different retention than their regular profiles, which are prepared in methanol.

Figure 5.3 and Figure 5.4 gives strong evidence to prove this guess. As it is shown, 5-OH-SIN shows a retention time of 11.293 min when it is in octanol, however, the retention time changed back to 11.820 min, which is regular profile, when diluted the octanol fraction 100 times into methanol. Similarly, 5-OH-HeptaMF, when in octanol, had a retention time of 11.493 min, whereas switched back to 14.407 min when diluted into methanol. These results mean the hydrogen bond between the hydrogen on 5-hydroxyl group and the oxygen on the 4-carbonyl group was broken in octanol, and reformed when octanol was removed.



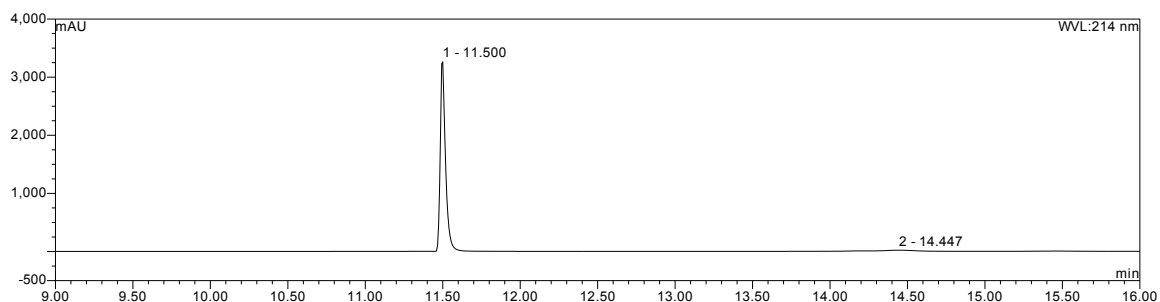


e

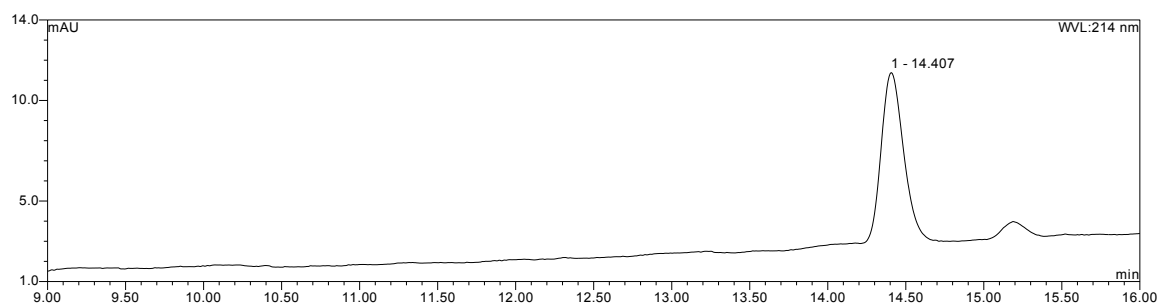


f

Figure 5.3 HPLC chromatogram of 5-OH-SIN in octanol (e) and octanol fraction diluted in methanol (f).



g



h

Figure 5.4 HPLC chromatogram of 5-OH-HeptaMF in octanol (g) and octanol fraction diluted in methanol (h).

In addition, as inspired by the HPLC chromatogram, 5-OH-PMFs with octanol present showed earlier retention time, which means they became more polar than their regular form. Therefore, octanol actually changed their hydrophilic affinity.

### 5.3.2 Partition coefficient (log P)

When a solute is added into a mixture of two immiscible liquids, the solute has a preference to distribute itself into the two liquids; when it reaches equilibrium, the ratio

of the two concentrations is called partition coefficient. The octanol-water partition coefficient is calculated via the following equation:

$$\log P = \log \left( \frac{\text{concentration in octanol}}{\text{concentration in water}} \right)$$

Table 5.1 Partition coefficient (log P) of PMFs and their derivatives. Each data represents mean  $\pm$  SEM (n=3). \*Data is lacking due to either no peak detected in water layer or peak area in water layer under reliable range.

PMFs	Partition coefficient (log P)
SIN	1.76 $\pm$ 0.22
5-OH-SIN	NA*
5-Ac-SIN	NA*
HeptaMF	1.97 $\pm$ 0.22
5-OH-HeptaMF	NA*
5-Ac-HeptaMF	NA*

The partition coefficient (log P) for SIN and HeptaMF are 1.76 and 1.97, respectively, which reflects a similar fact as HPLC chromatogram that HeptaMF is more lipophilic than SIN. Unfortunately, no partition coefficient was calculated for 5-OH-SIN and 5-OH-HeptaMF since there was no peak detected in the water layer at all. And for 5-Ac-SIN and 5-Ac-HeptaMF, their detected peak in water layer was too low and already under standard curve reliable range, therefore, their partition coefficient was not calculated as well.

## 5.4 Conclusion

In conclusion, the overall solubility of PMFs and their derivatives are very low. This *shake-flask* solubility test roughly tells us that the hydrophilic affinity of the tested PMFs is: SIN > 5-OH-SIN > 5-Ac-SIN, HeptaMF > 5-OH-HeptaMF > 5-Ac-HeptaMF, and SIN > HeptaMF, which is the same as the elution order from C<sub>18</sub> reverse phase HPLC system. The partition coefficient for SIN and HeptaMF are less than 5, which meets the Lipinski rule of five, indicating they qualify to be orally active drug. However, it is difficult to judge the 5-hydroxyl and 5-acetyl derivatives since their partition coefficient data is missing.

It was interesting to observe the hydrogen bond between the hydrogen on 5-hydroxyl group and the oxygen on the 4-carbonyl group break and re-form under different conditions. Since fatty acids have the same structural property as octanol – hydrophobic body and hydrophilic tail – 5-OH-PMFs might undergo the same change when mixed with fatty acids. Therefore, the hydrophilic affinity of 5-OH-PMFs is not as simple as predicted from in vitro study because our diet is made up of tens of hundreds of components, which could potentially change the form of administered compounds.

## **Chapter 6 Permeability Assessment of Polymethoxyflavones and Their Derivatives Through Caco-2 Cell Monolayer Transport Model**

### **6.1 Principle**

Polymethoxyflavones (PMFs), as the unique group of flavonoids in citrus peels, could potentially lead to the health benefits of aged orange peel. Numerous studies have been performed on PMFs indicating their anti-inflammatory (Gossiau, Chen, Ho, & Li, 2014; Ho, Pan, Lai, & Li, 2012), anti-carcinogenic (Lai et al., 2007), anti-oxidant (Teng, Yang, Chen, Lin, & Tsai, 2011), anti-atherogenic (Kurowska & Manthey, 2004), and hypolipidemic (Kurowska & Manthey, 2004) functions. However, lack of knowledge on their bioavailability slows down the understanding on this group of compounds.

Bioavailability of a drug or a nutrient is defined as the amount of the drug or the nutrient reaches the systemic circulation (Ting, Jiang, Ho, & Huang, 2014). Absorption is the first stage of bioavailability if the drug or nutrient is taken through oral administration.

Absorption is greatly influenced by two factors, one is solubility, which is discussed in Chapter 5, and the other one is permeability, which explains the process of drug moving across small intestine. The most well-known in vitro assay used to predict permeability is called Caco-2 cell monolayer transport model.

Caco-2 cells are the human colon carcinoma cell line. They are widely used in the prediction of drug permeability since they have enterocyte-like properties after forming a monolayer. Similarly to epithelial cells in small intestine, Caco-2 monolayer gives tight

junctions, mucosal/apical side, and serosal/basolateral side, along with various metabolic enzymes and transporters, such as sulfotransferase (Satoh, Matsui, & Tamura, 2000) and P-glycoprotein (Hunter, Jepson, Simmons, Hirst, & Tsuruo, 1993), respectively.

Therefore, Caco-2 monolayer would definitely be a powerful tool to unravel the mystery of a drug being transported in and out.

In this study, we investigated the permeability property of totally six PMFs and their derivatives, namely sinensetin (SIN), 5-demethyl-sinensetin (5-OH-SIN), 5-acetyl-sinensetin (5-Ac-SIN), 3,5,6,7,8,3',4'-Heptamethoxyflavone (HeptaMF), 5-hydroxy-3,6,7,8,3',4'-hexamethoxyflavone (5-OH-HeptaMF), and 5-acetyl-3,6,7,8,3',4'-hexamethoxyflavone (5-Ac-HeptaMF).

## **6.2 Experimental**

### **6.2.1 Materials**

SIN, HeptaMF, and 5-OH-HeptaMF were kindly provided by Dr. Shiming Li (Food Science Department, Rutgers, the State University of New Jersey, USA). 5-OH-SIN, 5-Ac-SIN, and 5-Ac-HeptaMF were chemically synthesized as mentioned in Chapter 4. Nobiletin (NOB) and 5-demethyl-nobiletin (5-OH-NOB) were obtained from the separation of orange peel extract mentioned in Chapter 3.

Caco-2 cell lines were obtained from Rutgers University. Dulbecco's modified essential medium (DMEM) high glucose with L-glutamine, MEM non-essential amino acids, trypsin-EDTA (0.5%), penicillin-streptomycin, phosphate-buffered saline-pH 7.2 (PBS), hank's balance salt solution (HBSS), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide were purchased from Gibco Life Technologies (Grand Island, NY, USA). Fetal bovine serum was purchased from Biowest (Kansas City, MO, USA). Dimethyl sulfoxide (DMSO) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Corning 96-well cell culture plates and 12-transwell plates were purchased from Fisher Scientific (Fairlawn, NJ, USA).

Acetonitrile (HPLC grade), water (HPLC grade), methanol (HPLC grade), and ethyl acetate (ACS grade) were purchased from Pharmco-AAPER (Brookfield, CT, USA).

### **6.2.2 Apparatus**

The HPLC system consisted of a Dionex UltiMate 3000 HPLC series (Sunnyvale, CA, USA) including an UltiMate 3000 Pump, an UltiMate 3000 Variable Wavelength Detector, and an UltiMate 3000 Auto-sampler. Chromeleon software was used to perform instrument control and data analysis. Supelco Ascentis® RP-Amide C18 HPLC column (15 cm X 4.6 mm, 3 µm) was purchased from Sigma-Aldrich (St. Louis, MO, USA).

The spectrophotometric microtiter plate reader was purchased from BioTek (Winooski, VT, USA).

### 6.2.3 Methods

#### 1). Cell Culture

Caco-2 cells (passage 53-63) were maintained in DMEM medium containing 10% FBS, 1% PS, and 1% non-essential amino acids. Cells were incubated in 5% CO<sub>2</sub> with 95% relative humidity at 37°C. Cells were detached by trypsin-EDTA treatment after reaching 80% confluence.

#### 2). MTT Assay

The aim of MTT assay in this study was to determine the proper dosage for PMFs and their derivatives, at which the concentration was applied in the Caco-2 monolayer transport experiment. The ultimate goal was to find one concentration, at which all the six PMFs and their derivatives are not harmful to the Caco-2 cells.

The screening process started with PMF concentration of 50 µM, and further went down to the concentration of 20 µM. To begin with, the PMF solutions were freshly prepared in DMSO and diluted into DMEM medium with the final DMSO concentration < 0.1%. Caco-2 cells were seeded into 96-well plates with a density of  $1 \times 10^5$  cells/well for 24 hours. Subsequently, cells were treated with 0.2 mL DMEM medium containing targeted concentration of PMF samples. The control group was treated with 0.2 mL DMEM



medium without PMFs added. After 24 hours, the DMEM medium was discarded, and the cells were washed with PBS. Subsequently, 0.2 mL 0.5 mg/mL MTT solution in DMEM medium was added into each well, and the cells were incubated for 4 hours. Finally, MTT solution was discarded, and the formazan crystals were extracted by 0.1 mL DMSO. Light absorption at 560 nm was measured by the spectrophotometric microtiter plate reader. Each PMF at each concentration was repeated six times. Data were expressed as mean  $\pm$  standard error mean (SEM). Statistical significance of mean difference between two groups was determined by student t-test. Significance levels of  $p < 0.05$  and  $p < 0.001$  were applied.

### 3). Caco-2 Cell monolayer Transport Assay

$3 \times 10^6$  cells were seeded onto the permeable inserts (0.4  $\mu\text{m}$  pore size and 12 mm diameter) of 12-transwell plates. The medium was changed every other day for 21 days to induce the differentiation of Caco-2 monolayer. The monolayer integrity was confirmed by measuring transepithelial electrical resistance (TEER) value at 37°C. Monolayers with TEER values lower than  $165 \Omega \text{ cm}^{-2}$  were discarded.

At the beginning of the transport experiment, cells were washed twice with pre-warmed HBSS solution and then incubated for 20 min at 37°C. For apical (AP) to basolateral (BL) transport, 0.45 mL HBSS containing 20  $\mu\text{M}$  PMF samples was added to the AP side, meanwhile 1.2 mL blank HBSS was added to the BL side. Similarly, for BL to AP transport, 1.25 mL HBSS containing 20  $\mu\text{M}$  PMF samples was added to the BL side, and

0.4 mL blank HBSS was added to the AP side. For both, 0.05 mL solution was taken at 0 min from the donor chamber to confirm the initial concentration. The monolayers were incubated at 37°C, and sample solutions (0.6 mL for AP-BL and 0.2 mL for BL-AP) were taken at 20, 40, 60, 80, and 100 min from the receiver chambers and immediately re-added the same amount of blank HBSS solution to maintain the volume of 1.2 mL for AP-BL and 0.4 mL for BL-AP.

#### 4). Sample analysis

Each sample collected from the transport experiment was added with 0.1 mL 20  $\mu$ M NOB or 5-OH-NOB in HBSS as internal standard, and extracted with 0.6 mL ethyl acetate twice. The EA layer was collected and evaporated in oven at 40°C. Then 0.1 mL methanol was used to reconstitute the residue.

The HPLC program is as following:

The mobile phase consists of water (A) and acetonitrile (C); and the 20 min gradient starts with 40% C, then linearly increases to 55% C in 10 min, and then linearly increases to 70% C in 15 min, finally stays at 80% C at 20 min; then increases to 100% C in 21 min and keeps there for 4 min to wash the column, and then goes back to 40% C for equilibrium. Data was collected at wavelength of 214 nm, 254 nm, and 326 nm. The injection volume was set at 50  $\mu$ L, and the flow rate was 1 mL/min.

The concentration of each sample was calculated from its correlated standard curve. Data were expressed as mean  $\pm$  standard error mean (SEM).

## 6.3 Results and Discussion

### 6.3.1 MTT assay

The relative cell viability was calculated by using the following equation:

$$Cell\ viability\ (\%) = \frac{OD(560\ nm, sample) - OD(560\ nm, blank)}{OD(560\ nm, control) - OD(560\ nm, blank)} \times 100\%$$

(Duan et al., 2014)

The viability of the control group was considered 100%. By screening down from 50  $\mu$ M to 20  $\mu$ M, it was found that 20  $\mu$ M was the best concentration to go with. Above 20  $\mu$ M concentration, some compounds, like 5-OH-HeptaMF, had the viability below 80%.

However, the cell viability was above 95% for all the PMF samples at 20  $\mu$ M concentration, meaning PMFs are not cytotoxic at this concentration (Figure 6.1).

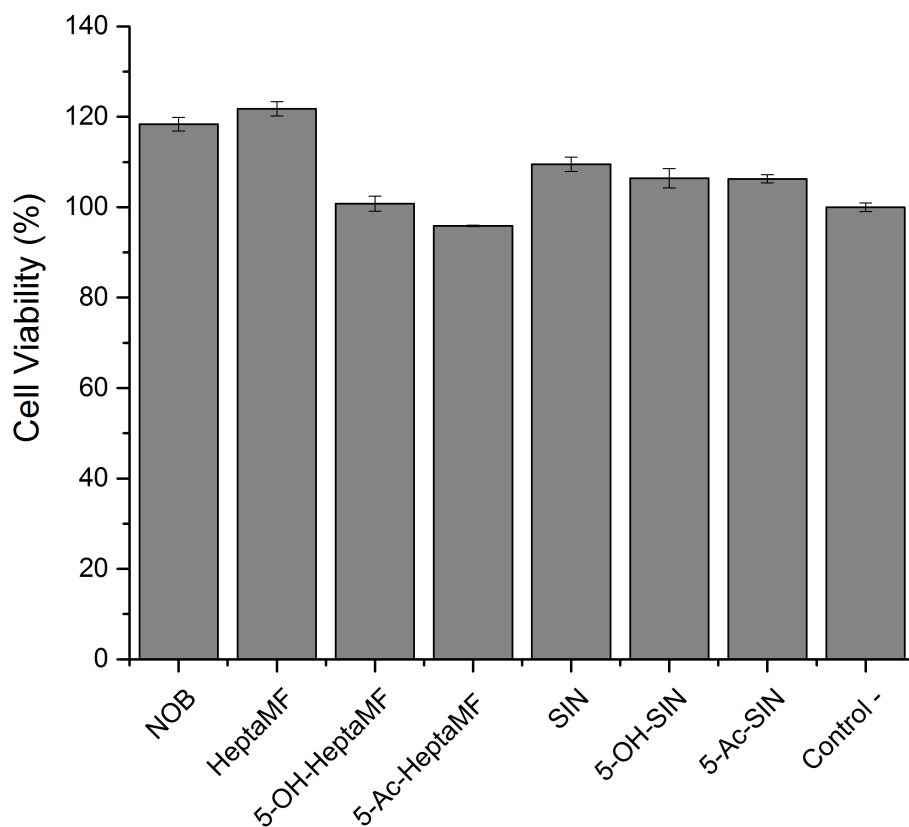


Figure 6.1 Caco-2 cell viability after treated with PMFs and their derivatives at a concentration of 20  $\mu$ M. Each bar was expressed as mean  $\pm$  SEM (n=6).

Thus, 20  $\mu$ M was chosen as the dosage for the Caco-2 monolayer transport experiment.

Except for achieving this purpose, MTT assay also gave indication on the inhibitory effects of PMFs and their derivatives on human colon cancer cells. In this section, comparison on the inhibitory effects of the HeptaMF series is discussed.

As it is shown in Figure 6.2, as concentration increases, the relative cell viability decreases no matter treated with which compound. 50  $\mu$ M was the biggest dosage used in this study, which gave 92.52, 70.34, and 60.31% viable cells after treated with HeptaMF, 5-OH-HeptaMF, and 5-Ac-HeptaMF, respectively. At  $p < 0.001$  level, cell viability

treated with 5-OH-HeptaMF and 5-Ac-HeptaMF was significantly different from control group, while it was not significantly different when treated with HeptaMF. This result reveals that 5-OH-HeptaMF and 5-Ac-HeptaMF are more powerful than HeptaMF in the inhibition of human colon cancer cells. In addition, cell viability treated with 5-Ac-HeptaMF was significantly greater than treated with 5-OH-HeptaMF ( $p < 0.05$ ), which means, with the addition of an acetyl group, the anti-cancer property is promoted. Generally speaking, 5-Ac-HeptaMF had the best inhibitory effect, followed by 5-OH-HeptaMF and HeptaMF.

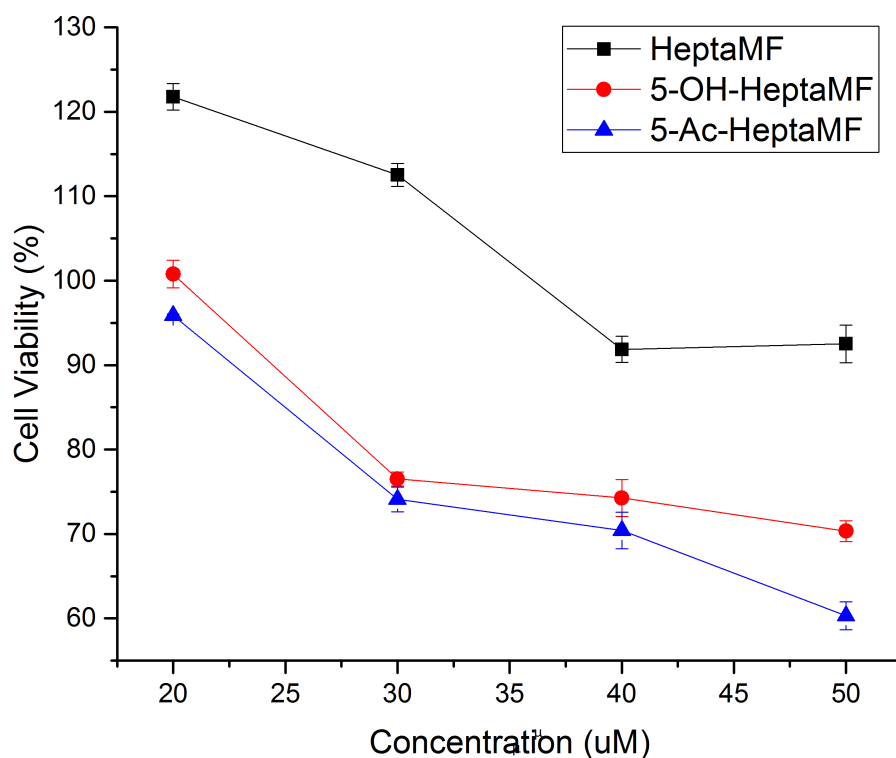


Figure 6.2 Inhibitory effects of HeptaMF, 5-OH-HeptaMF, and 5-Ac-HeptaMF on Caco-2 cells. Each point represents mean  $\pm$  SEM ( $n=6$ ).

### 6.3.2 Caco-2 Cell monolayer Transport Assay

In order to systemically compare the permeability properties of PMFs and their derivatives, Caco-2 monolayer transport experiment was carried out on SIN, 5-OH-SIN, 5-Ac-SIN, HeptaMF, 5-OH-HeptaMF, and 5-Ac-HeptaMF, and the experiment was conducted for both apical to basolateral (AP-BL) and basolateral to apical (BL-AP) direction. In order to present the amount of compound permeated, we calculated permeability rate (%) using this following equation:

$$\text{Permeability Rate} = \frac{\text{Accumulated } \mu\text{mol (t)}}{\text{original } \mu\text{mol}} \times 100\%$$

Data were expressed as mean  $\pm$  SEM. Figure 6.3 shows the permeability rate of all the tested compounds at each time point. There are several trends can be summarized:

1) PMFs had better permeability than their derivatives in the AP-BL direction.

At 100 min from AP to BL, SIN had 48.78% permeated, while 5-OH-SIN and 5-Ac-SIN had 6.54% and 27.70% permeated, respectively. Similarly, HeptaMF had 45.89% permeated at 100 min from AP to BL, but only 6.84% for 5-OH-HeptaMF and 6.56% for 5-Ac-HeptaMF. This means it is easier for PMFs to enter the systemic circulation than their 5-hydroxyl and 5-acetyl derivatives.

2) PMFs had absorption over efflux, while the derivatives had efflux over absorption.

At 100 min, SIN had 39.51% more absorbed than pumped out; however, 5-OH-SIN and 5-Ac-SIN had 11.03% and 3.35% more pumped out than absorbed, respectively. Similarly, HeptaMF had 3.26% more absorbed than pumped out, while 14.41% and 27.81% more pumped out for 5-OH-HeptaMF and 5-Ac-HeptaMF, respectively. It was indicated that PMFs are more likely taken into the system; on the contrary, their 5-hydroxyl and 5-acetyl derivatives tend to be pumped out very fast from the blood circulation system.

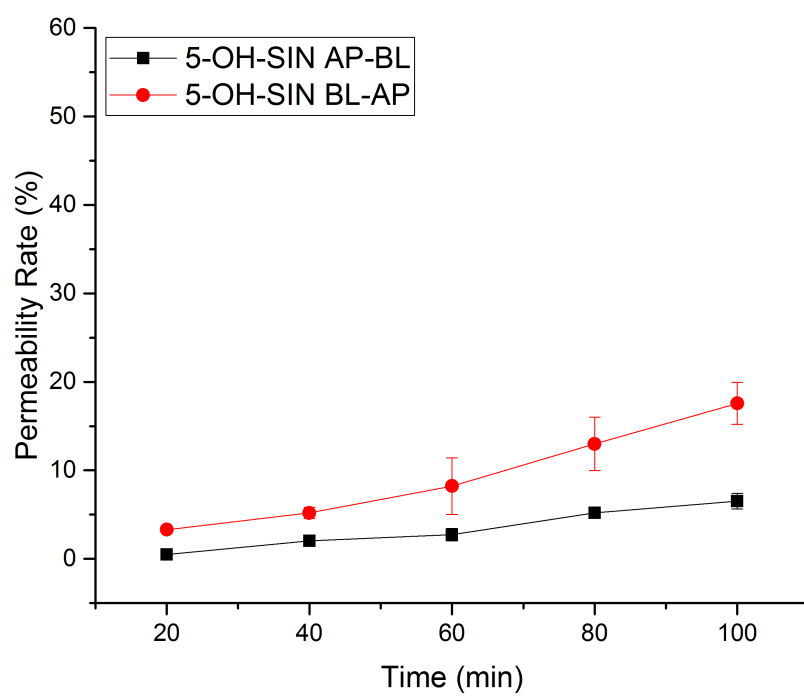
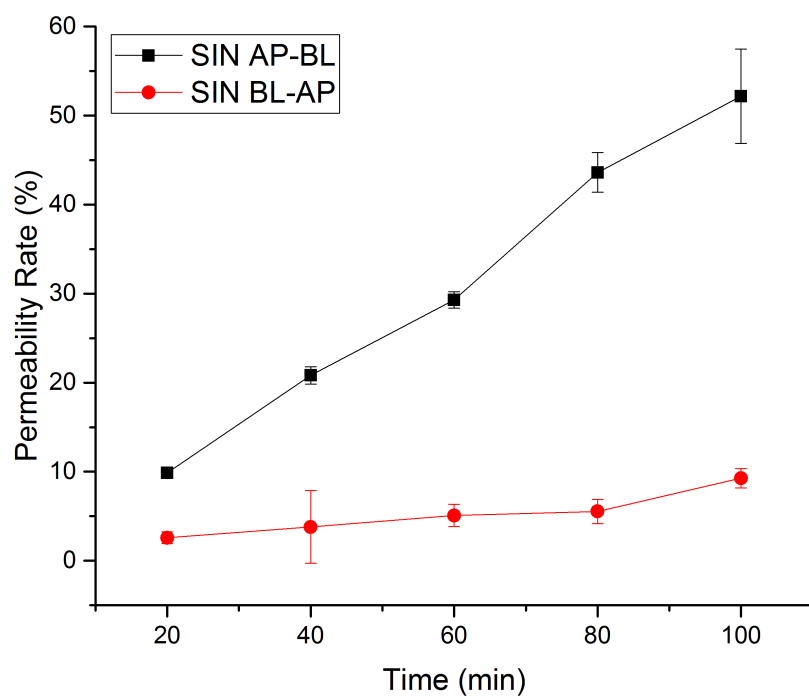
### 3) SIN vs. HeptaMF

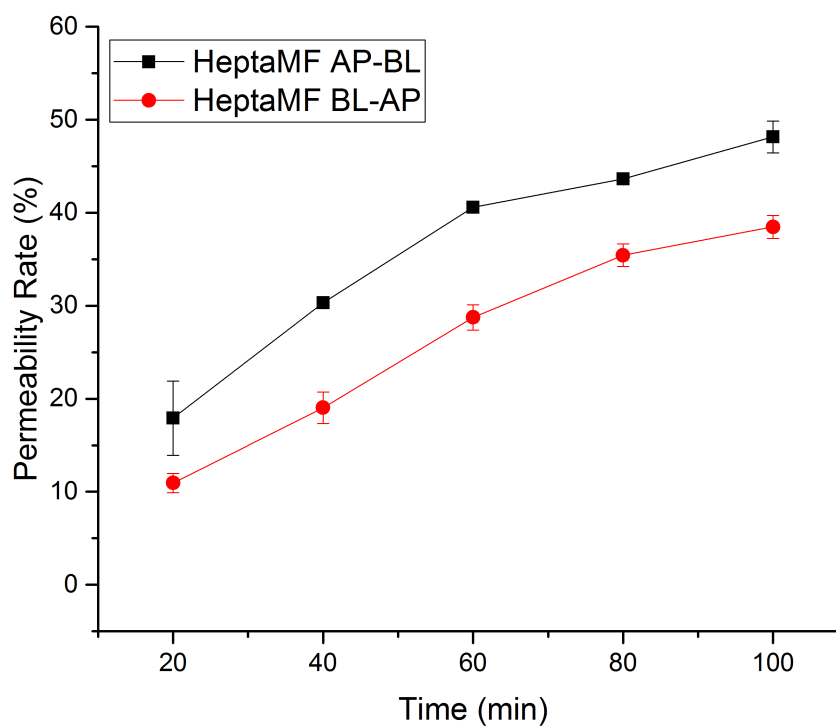
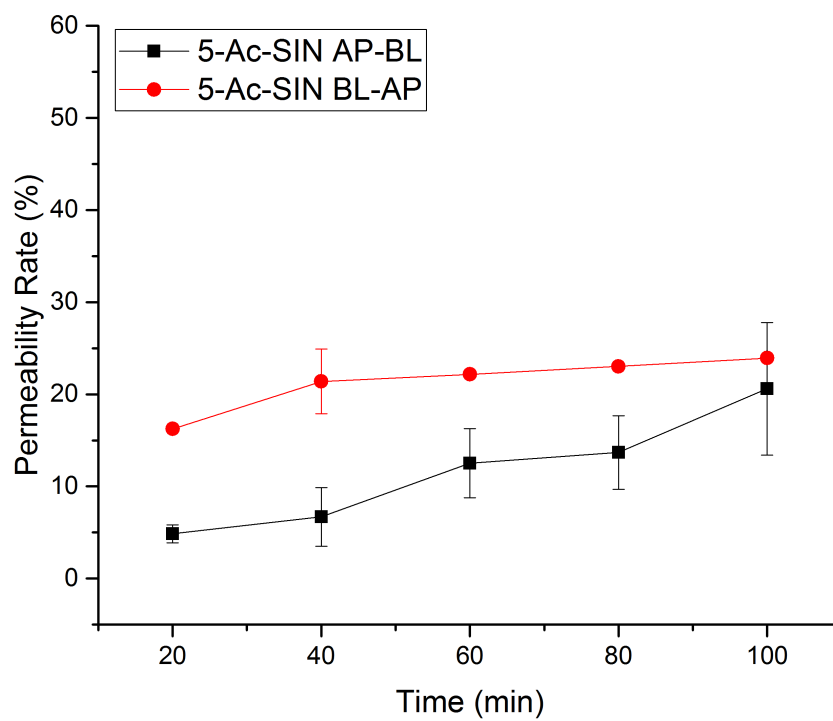
There was no big difference between SIN and HeptaMF on the absorption direction. However, by comparing the efflux direction, SIN had only 9.27% pumped out, while HeptaMF had 42.64%. It tells that SIN can be easily absorbed but hard to leave the system; by contrast, it is easy for HeptaMF to enter as well as leave the systemic circulation.

4) 5-Acetyl-PMFs as pro-drugs successfully improved permeability of 5-hydroxy-PMFs.

5-Hydroxy-PMFs was reported having more potent biological activities than their PMF counterparts especially anti-inflammation and anti-cancer (Lai et al., 2007). The idea of synthesizing 5-acetyl-PMFs was to deliver 5-hydroxy-PMFs into the system and better perform the biological functions. 5-Ac-SIN had a permeability rate of 20.59% (AP-BL) and 23.94% (BL-AP), which were greater than 6.54% (AP-BL) and 17.57% (BL-AP) for 5-OH-SIN. Furthermore, 5-Ac-HeptaMF had 6.56% (AP-BL) and 34.37% (BL-AP) transported, while 5-OH-HeptaMF had 6.84% (AP-BL) and 21.26% (BL-AP) transported. However, the permeability rates of 5-Ac-SIN and 5-Ac-HeptaMF were underestimated since metabolic changes were not taken into consideration here. Details on metabolism are discussed in the following section.







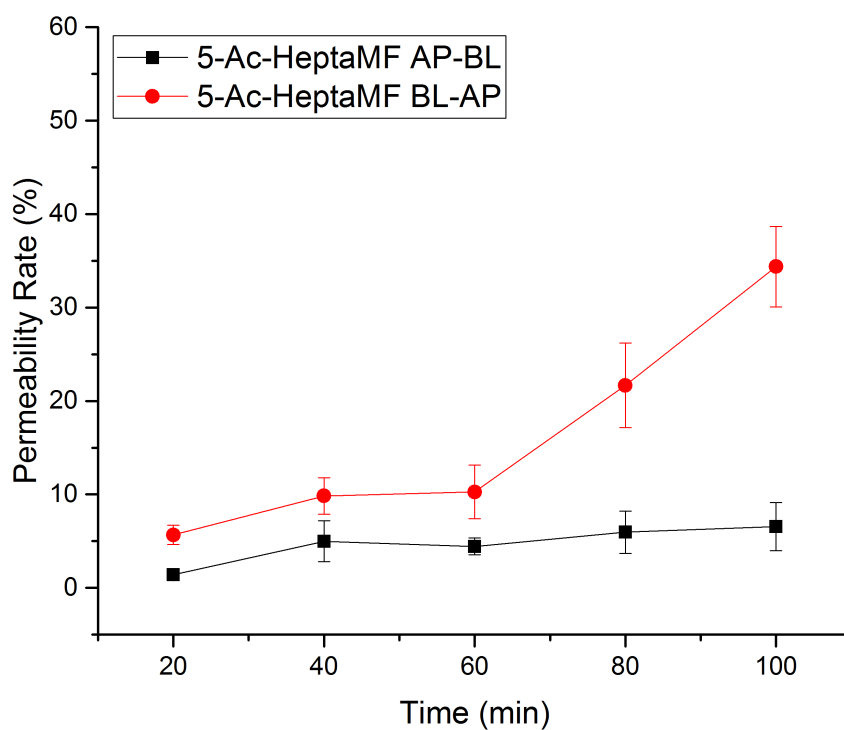
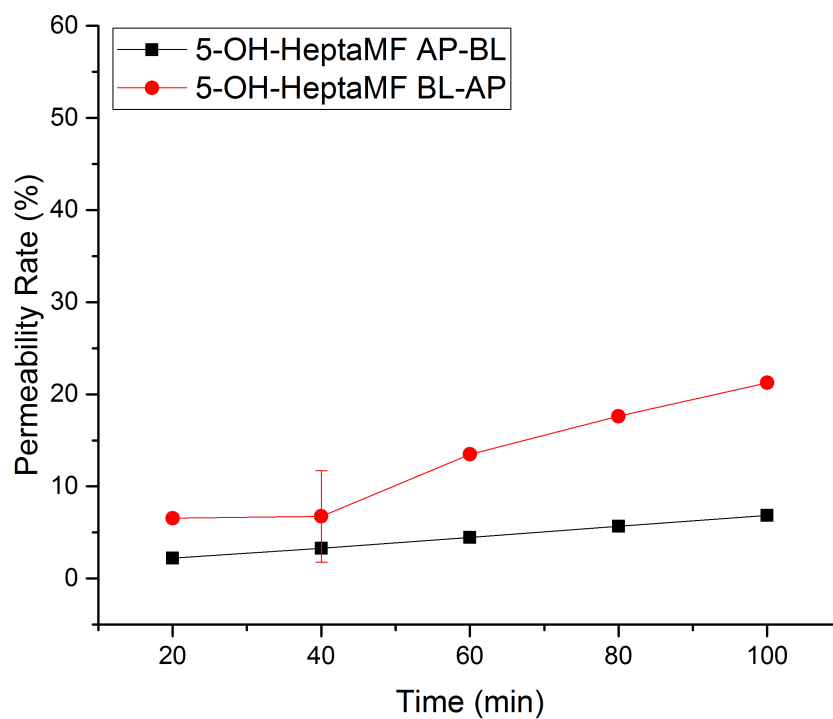


Figure 6.3 Permeability rates of PMFs and their derivatives as a function of time. Each point represents mean  $\pm$  SEM (n=3).

### 6.3.3 Apparent permeability coefficient and efflux ratio

The Apparent permeability coefficient ( $P_{app}$ ,  $\text{cm s}^{-1}$ ) measures the amount of drug transported per time:

$$P_{app} = \frac{dQ}{dt} \times \frac{1}{AC_0}$$

Where  $dQ/dt$  is the steady state flux ( $\mu\text{mol s}^{-1}$ ),  $A$  is the surface area of the permeable insert ( $\text{cm}^2$ ), and  $C_0$  is the initial concentration ( $\mu\text{M}$ ) (Hubatsch, Ragnarsson, & Artursson, 2007). Data were calculated using the linear range where the receiver chamber has  $< 10\%$  of the loading concentration to satisfy the sink condition, which means the back diffusion of drug from the receiver chamber is negligible (Sun & Pang, 2008).

Efflux ratio (EfR) is used to compare the drug absorption and efflux:

$$EfR = \frac{P_{app} (BL - AP)}{P_{app} (AP - BL)}$$

(Sun & Pang, 2008)

Table 6.1 Apparent permeability coefficient and efflux ratio of tested PMFs and their derivatives.

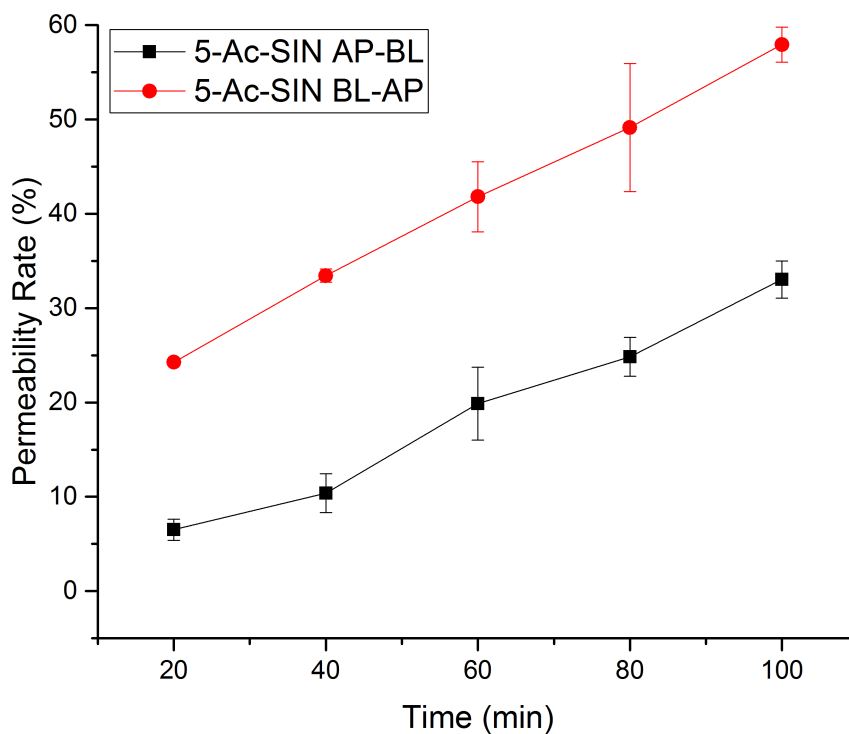
	<b>Papp (AP-BL)</b> <b>cm s<sup>-1</sup> × 10<sup>-7</sup></b>	<b>Papp (BL-AP)</b> <b>cm s<sup>-1</sup> × 10<sup>-7</sup></b>	<b>EfR</b>
<b>SIN</b>	1042	47	0.05
<b>5-OH-SIN</b>	108	138	1.28
<b>5-Ac-SIN</b>	543	794	1.46
<b>HeptaMF</b>	1091	320	0.29
<b>5-OH-HeptaMF</b>	133	138	1.04
<b>5-Ac-HeptaMF</b>	132	190	1.44

Generally, a compound has good permeability when  $P_{app} > 100 \times 10^{-7}$  cm/s, and efflux liability shows up when  $EfR > 3$  (Li et al., 2009). By looking through the data presented in Table 6.1, it was found that they all had good permeability except for SIN at the efflux direction ( $P_{app} = 47 \times 10^{-7}$  cm/s). In addition, SIN at the absorption direction, 5-Ac-SIN, and HeptaMF at both directions had much higher  $P_{app}$  than  $100 \times 10^{-7}$  cm/s, indicating they have excellent permeability.  $EfR$  values for all the tested compounds were below 3, which means no efflux liability for those compounds.

#### 6.3.4 Metabolism

Various enzymes were previously reported being present in the Caco-2 cell monolayer, which can metabolize certain compounds while they are transporting through the monolayer. For example, methylation and sulfation were observed on green tea catechins from the Caco-2 monolayer transport experiment (Zhang, Zheng, Chow, & Zuo, 2004).

In this study, we observed deacetylation from the transport of 5-Ac-SIN and 5-Ac-HeptaMF from the HPLC analysis. Deacetylation was found on 5-Ac-SIN from both AP-to-BL and BL-to-AP direction, but only detected from AP-to-BL transport for 5-Ac-HeptaMF. Therefore, in Figure 6.3, the permeability rates of 5-Ac-SIN and 5-Ac-HeptaMF were underestimated. After adjusting the amount of deacetylated, Figure 6.4 gives the total amount of 5-Ac-SIN and 5-Ac-HeptaMF permeated in this experiment.



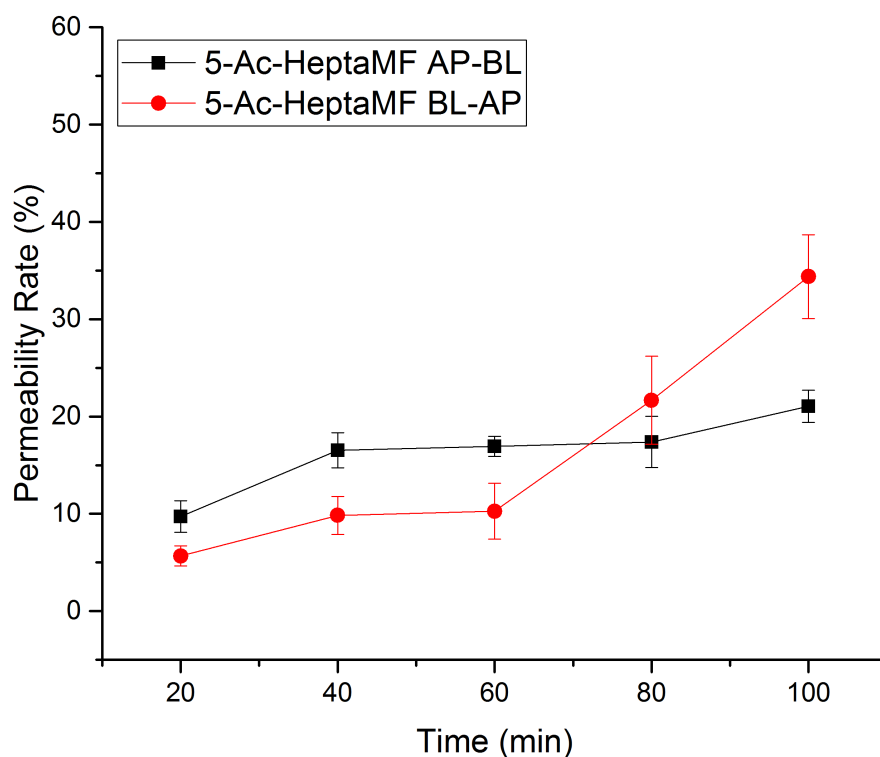


Figure 6.4 Total permeability rates of 5-Ac-SIN and 5-Ac-HeptaMF with the amount of deacetylated added. Each point represents mean  $\pm$  SEM (n=3).

For 5-Ac-SIN at 100 min, 33.03% (AP-BL) and 57.94% (BL-AP) permeated, which was much more than calculated before adjusting the amount of deacetylated (20.59% and 23.94%, respectively). And 5-Ac-HeptaMF had actually 21.05% (AP-BL) permeated other than 6.56%. By adding up the amount of deacetylated, it is clear to see that these 5-acetyl PMFs had much better permeability than their 5-hydroxyl PMFs, which further proved that 5-acetyl-PMFs are good pro-drugs to deliver 5-hydroxy-PMFs.

## 6.4 Conclusion

Polymethoxyflavones have well-known biological functions, including anti-cancer, anti-inflammation, anti-oxidant, and so on. It will benefit if we can fully understand bioavailability of this unique group of compounds. In order to make a contribution to research on the bioavailability of PMFs, this study systemically compared the permeability of PMFs, 5-hydroxyl-PMFs, and 5-acetyl-PMFs by applying the Caco-2 cell monolayer transport model.

Overall, PMFs and their tested derivatives have good permeability. The idea of pro-drug should definitely be promoted since 5-Ac-PMFs have better permeability than 5-OH-PMFs. Metabolism, especially deacetylation, was observed in this experiment. HPLC was used as the only detecting tool in our study; however, because the analyzing level is very low and UV detection is not sensitive enough, this could potentially result in big error in our results. More sensitive analytical methods, such as LC-MS, are needed to obtain more accurate data.

Transport pathway of PMFs was not investigated, but highly needed in order to grasp the whole picture of their bioavailability. Furthermore, in-vivo studies are of great interest to test the accuracy of this Caco-2 cell monolayer transport model.



## Chapter 7 Conclusion and Future Works

Even though there have been numerous studies conducted on polymethoxyflavones, it is still a long way to go in terms of fully understanding this unique group of flavonoids. To conclude this study, I would like to summarize the major findings as well as some future works that can boost the research on PMFs and their derivatives.

### 1). Database of PMF compositions from different sources

PMFs exist exclusively in citrus peels, especially in the peels of tangerine/mandarin and orange. Citrus juice industries yield huge volume of peels as the by-product each year, and most of the peels are simply sold as a source of pectin. In Asian countries, especially China and Japan, small amount of orange peels are collected and made into aged orange peels, also called Chenpi, which can be easily found in local stores. In the market, price for Chenpi varies from location to location. Xinhui Chenpi from Guangdong province is said the most valuable Chenpi in China, which sometimes costs several thousand dollars per kilogram. We compared the PMF composition of Jiangxi Chenpi with Guangdong Chenpi using HPLC system, and found that Guangdong Chenpi has more 5-hydroxylated PMFs, which is probably the reason why it has better treatment effects over other Chenpi. Meanwhile, other hydroxylated PMFs at C3', C4', and C7 positions should also be taken into consideration while evaluating the total PMF composition. It is crucial to have a well-established PMF database based on different citrus sources, such as species, planting

conditions, and years of storage, in order to value the market as well as to make the best of citrus peel by-products.

## 2). Bioavailability of PMFs

In this study, we investigated the solubility and permeability of PMFs from in vitro studies. Overall speaking, PMFs have poor solubility but high permeability. The result gives indicative information on the bioavailability of PMFs.

With knowing the poor solubility of PMFs, one key is to find a way to improve the solubility by chemical modification, delivery system, or any other methods. Our solubility experiment observed the changes of hydrophilic affinity of 5-OH-PMFs under certain conditions. Thus, inspired by this phenomenon, we could see the importance of finding out the mechanism of hydrophilic affinity changes, and thinking about the applications in functional food or nutraceutical industries.

Caco-2 cell monolayer transport model is a gold standard to test permeability of certain drugs. However, we have not made full use of this model yet. According to previous research, what make Caco-2 cell monolayer the best candidate to evaluate permeability are not only the differentiated tight junctions, apical and basolateral sides, but also more valuable are the protein transporters it can express. By studying on the transporters, the transport mechanism of PMFs will be gradually unraveled. Future study will be focused on studying the transport mechanism without a doubt. On the other hand, in vivo animal

study has to be carried out on PMFs to confirm the accuracy of Caco-2 cell monolayer model. Meanwhile, in vivo study gives detailed information on the bioavailability facts, including permeability through small intestine, concentration in serum, localization in organs, metabolism, secretion, and so on.

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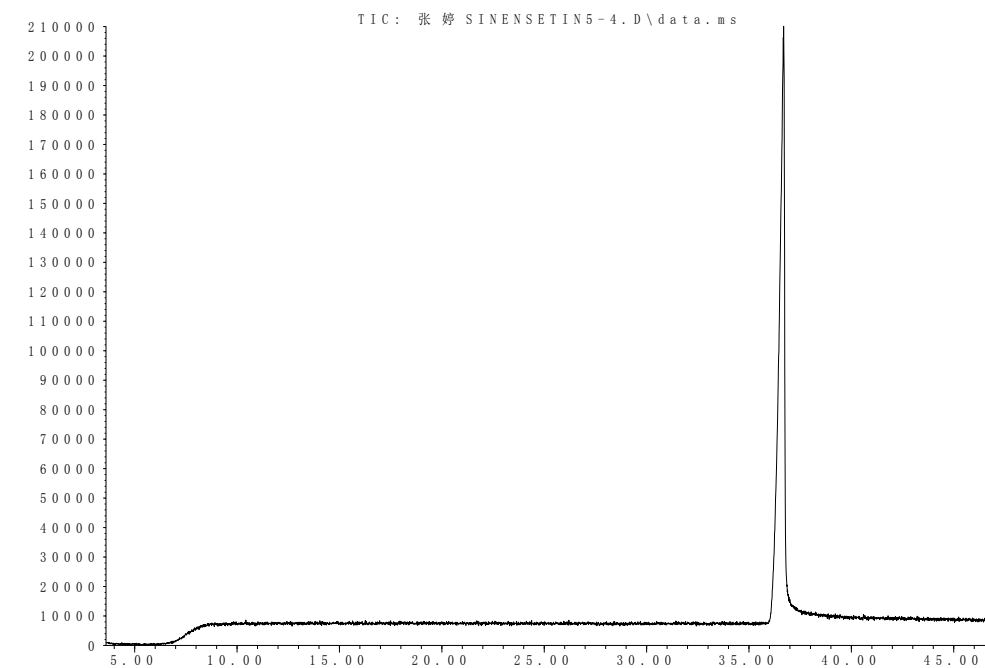
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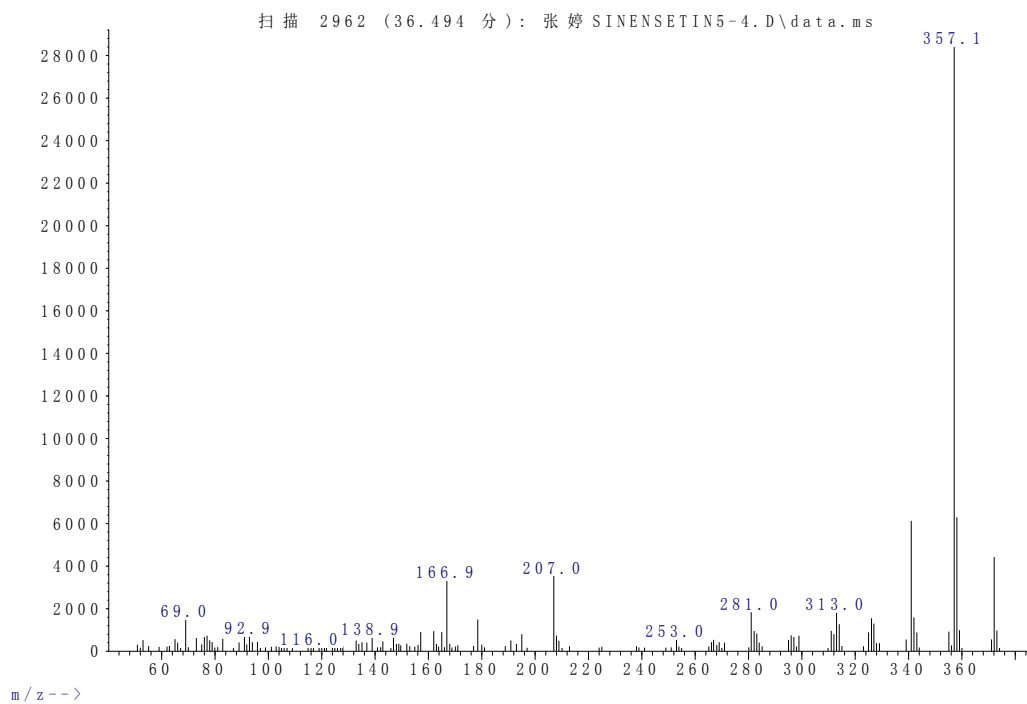
## Appendix A – GC-MS Graphs

### 1). SIN

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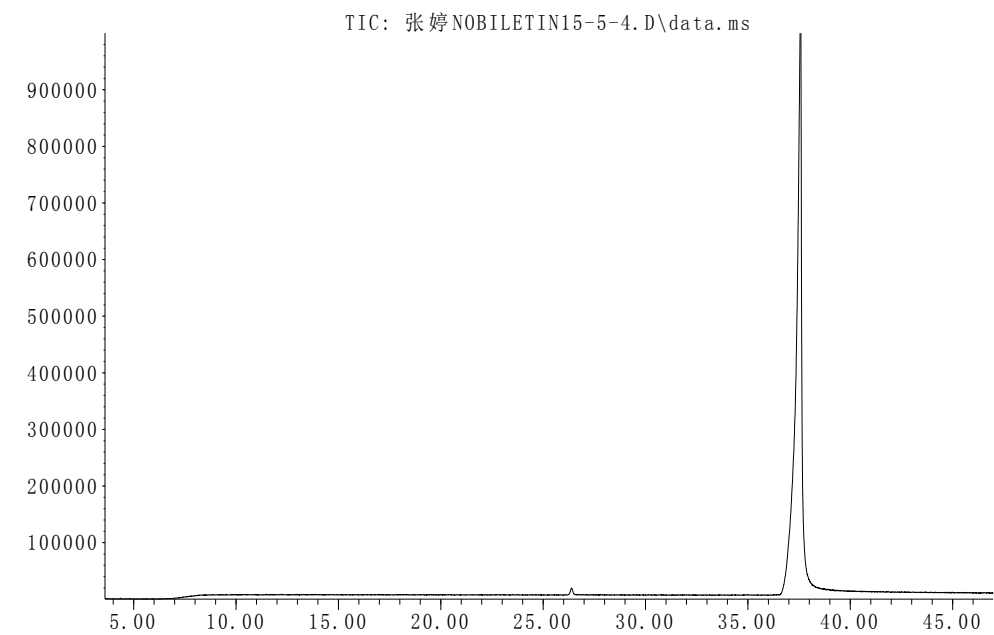
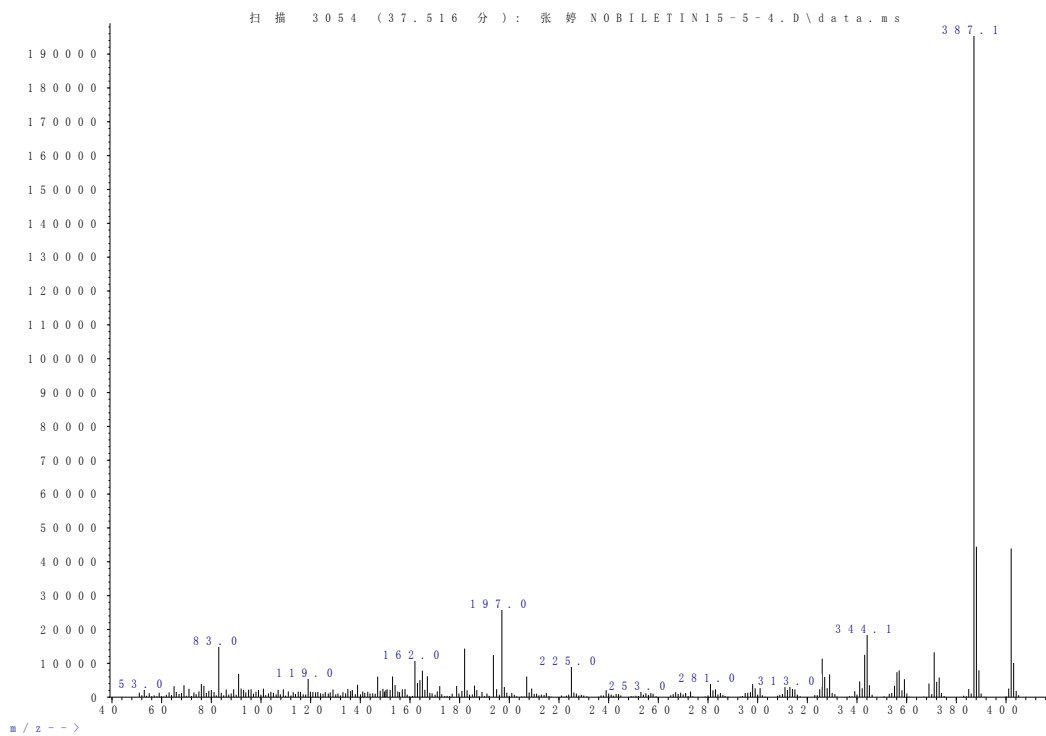


时间 -->  
丰度



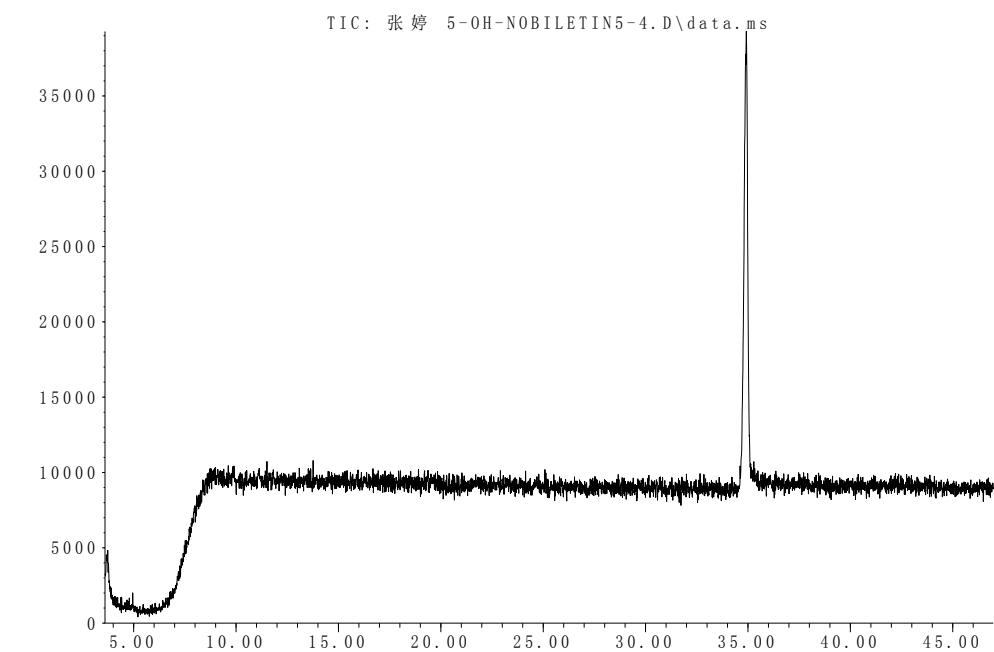
## 2). NOB

丰度

时间-->  
丰度

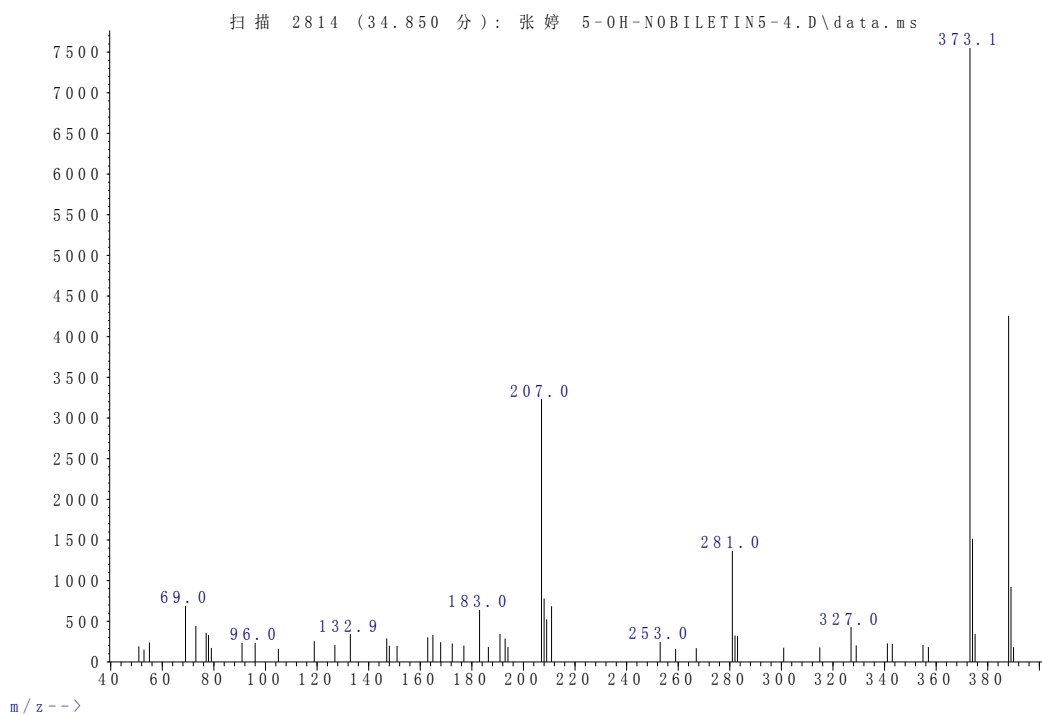
## 3). 5-OH-NOB

丰度



时间--&gt;

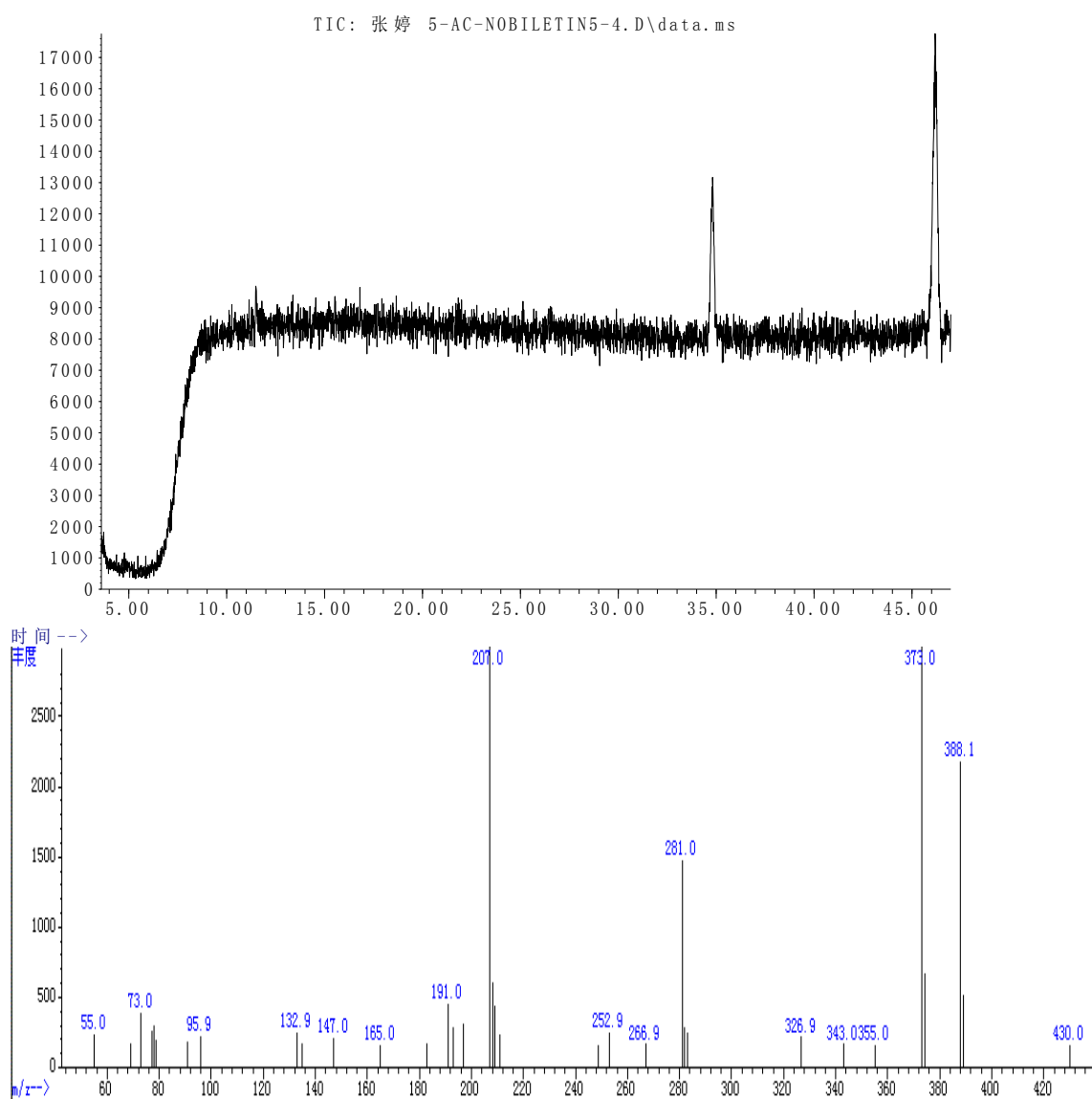
丰度



m/z--&gt;

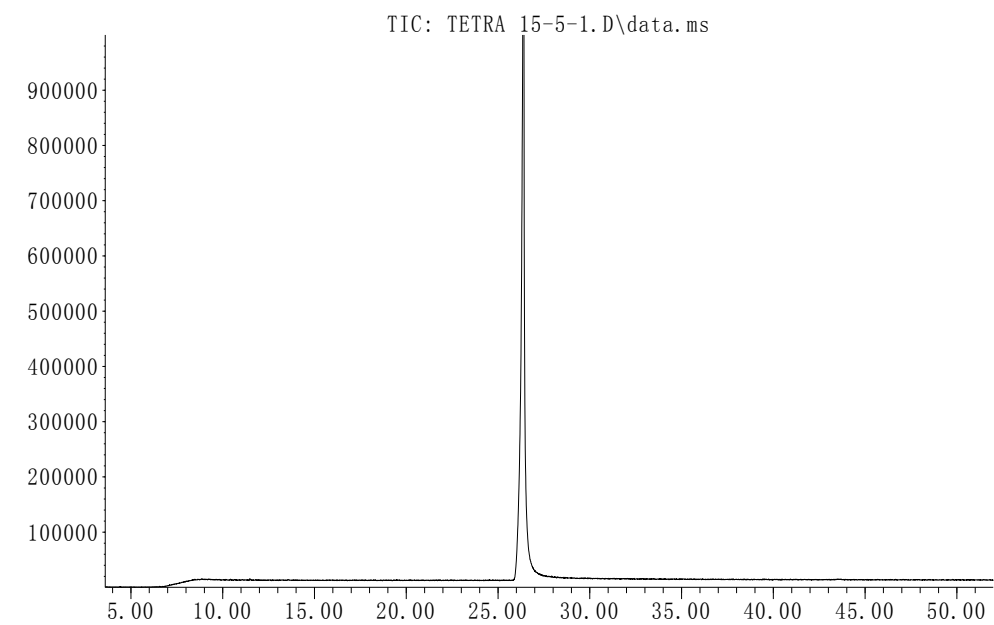
## 4). 5-Ac-NOB

丰度



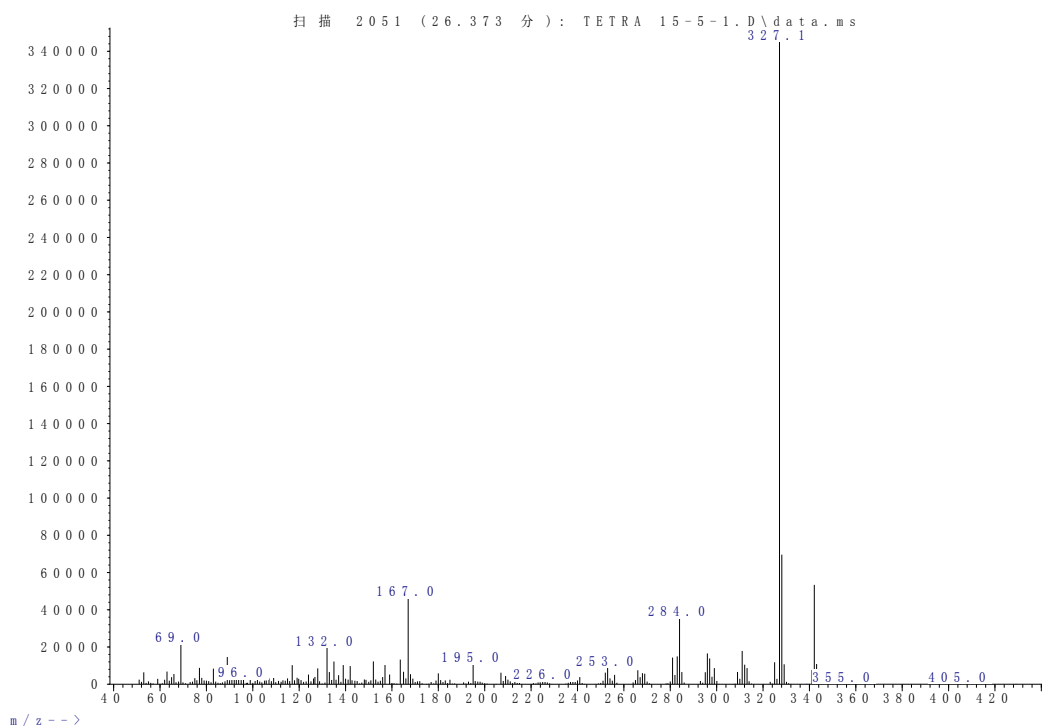
## 5). TetraMF

丰度



时间--&gt;

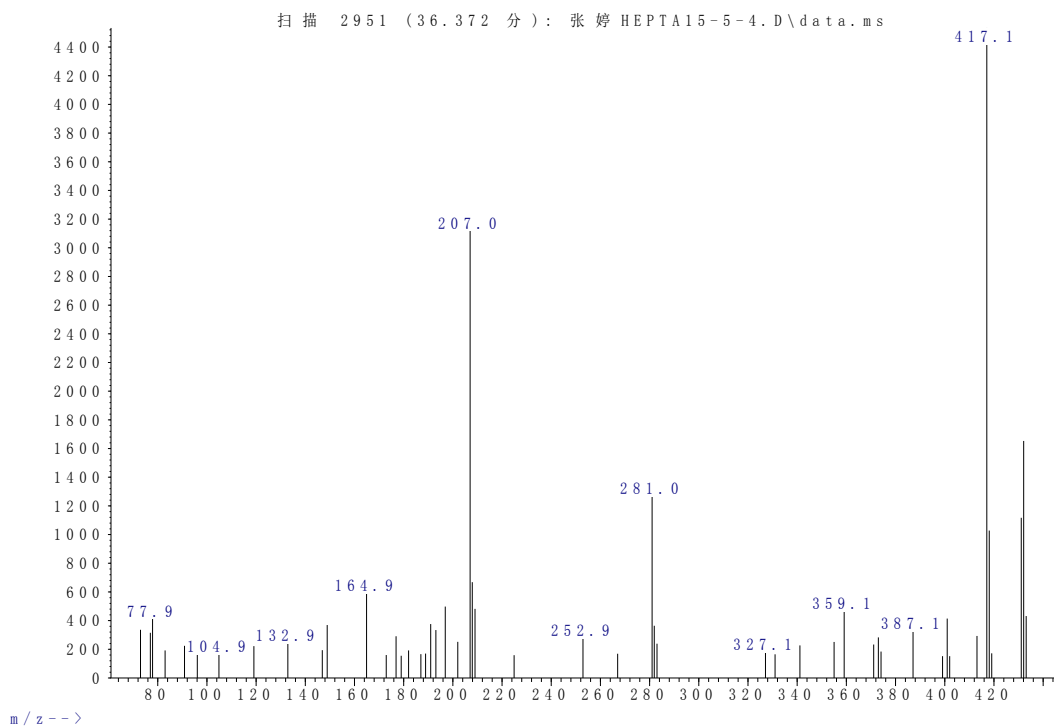
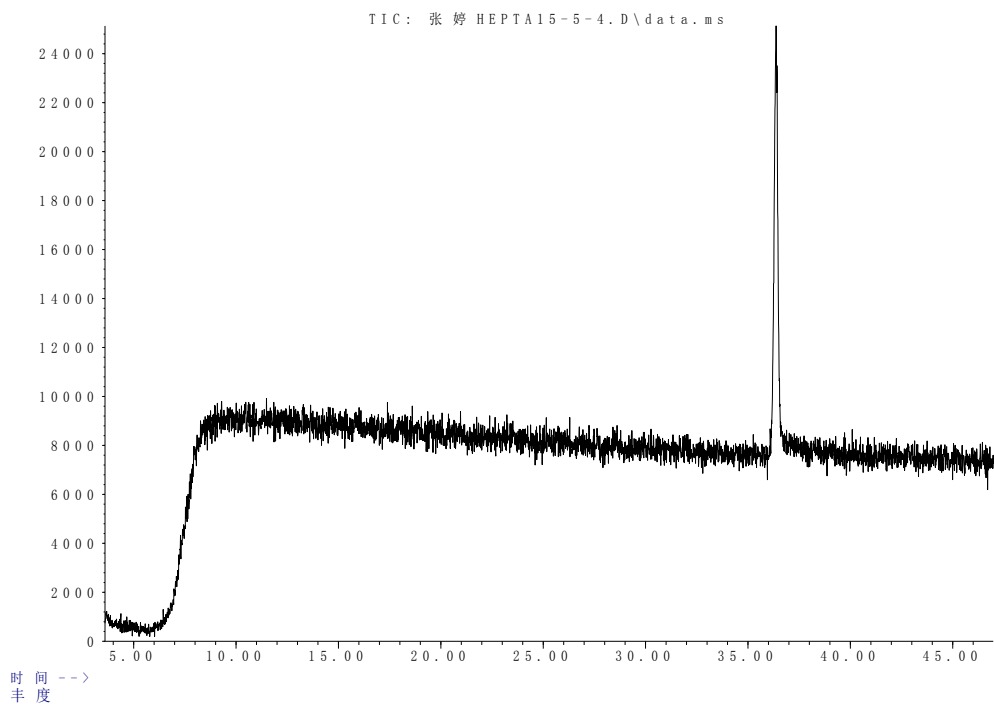
丰度



m/z--&gt;

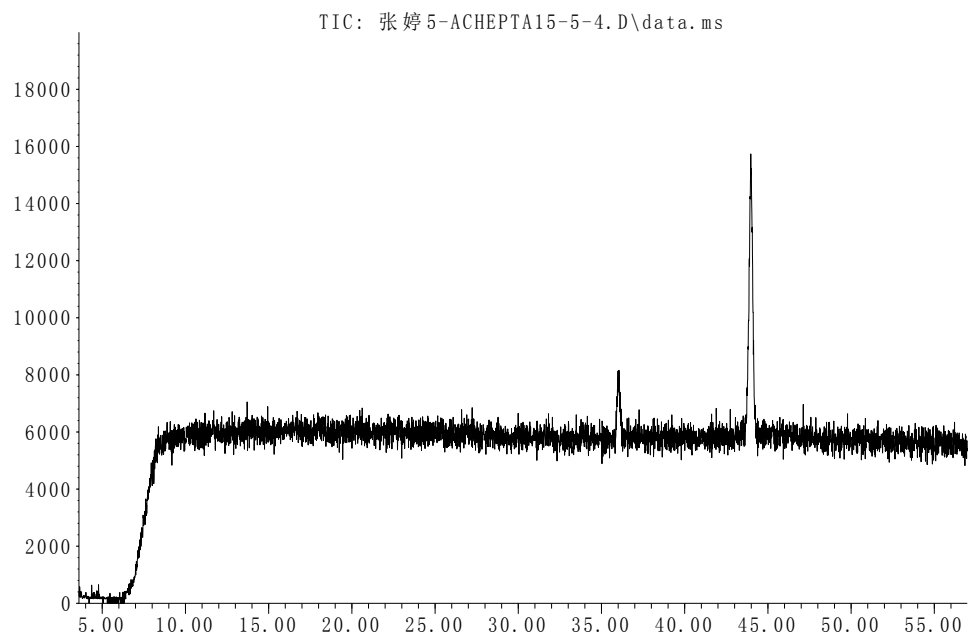
## 6). HeptaMF

丰度

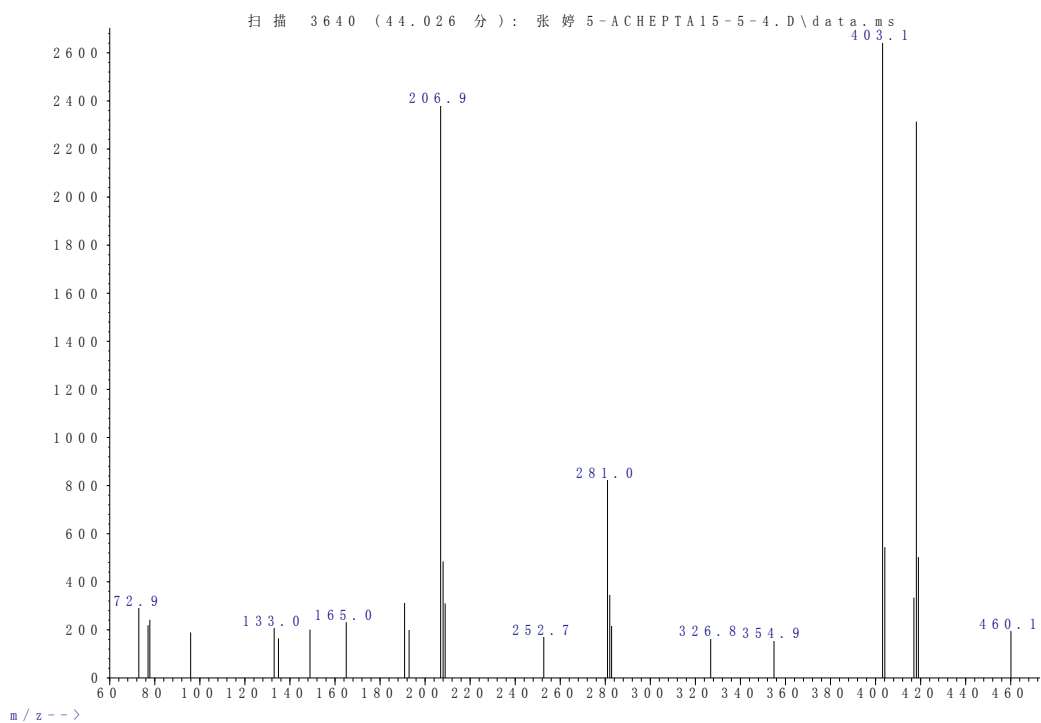


## 7). 5-Ac-HeptaMF

丰度

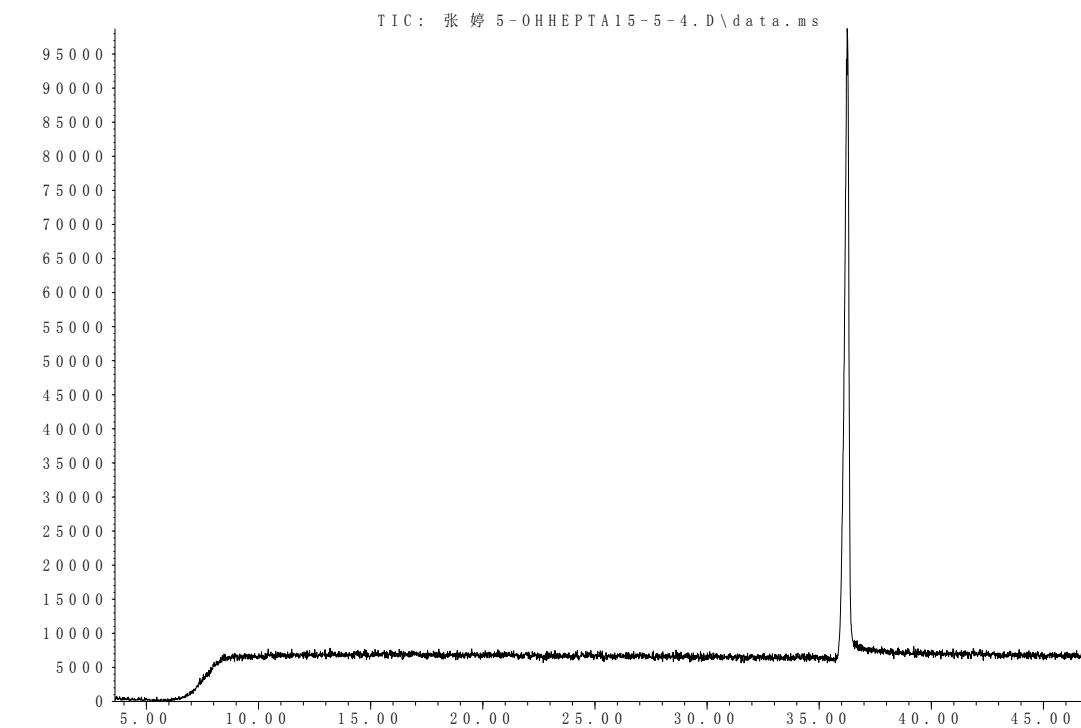
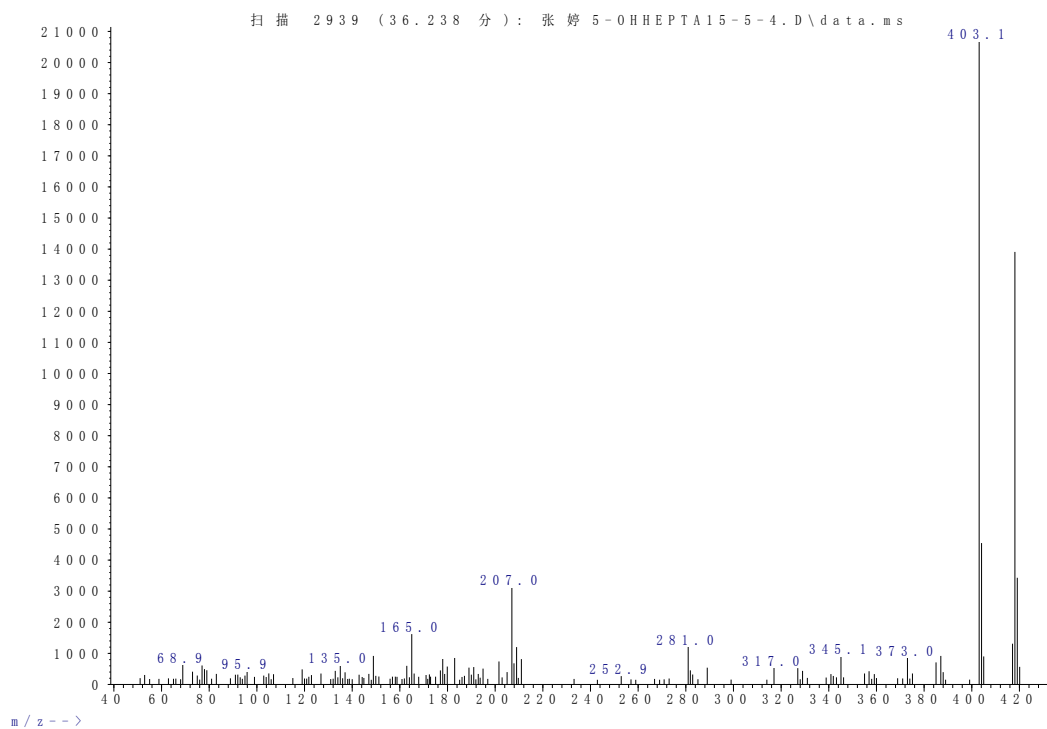


丰度



## 8). 5-OH-HeptaMF

丰度

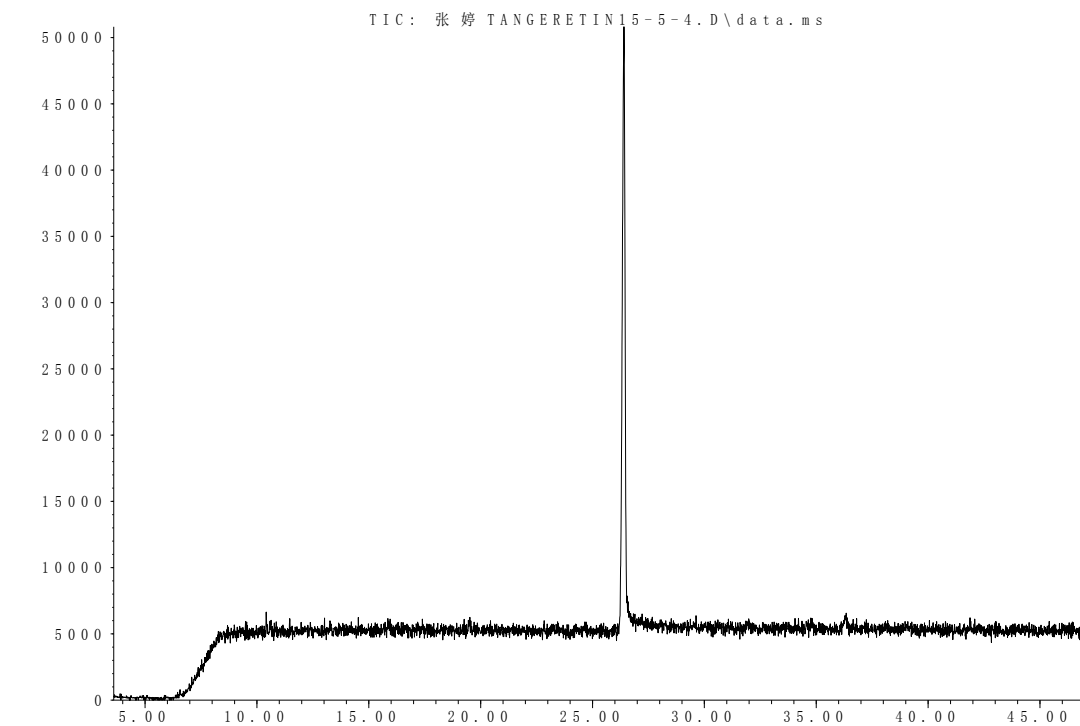
时间 -->  
丰度

m/z --&gt;

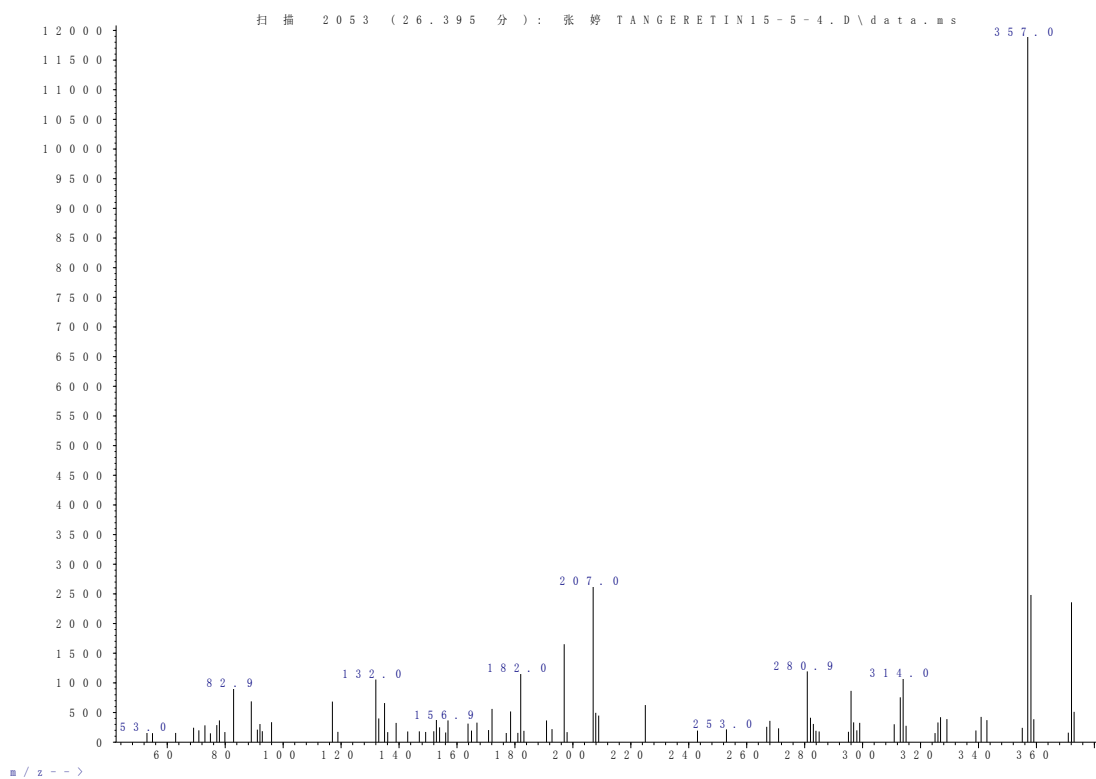


## 9). TAN

丰度

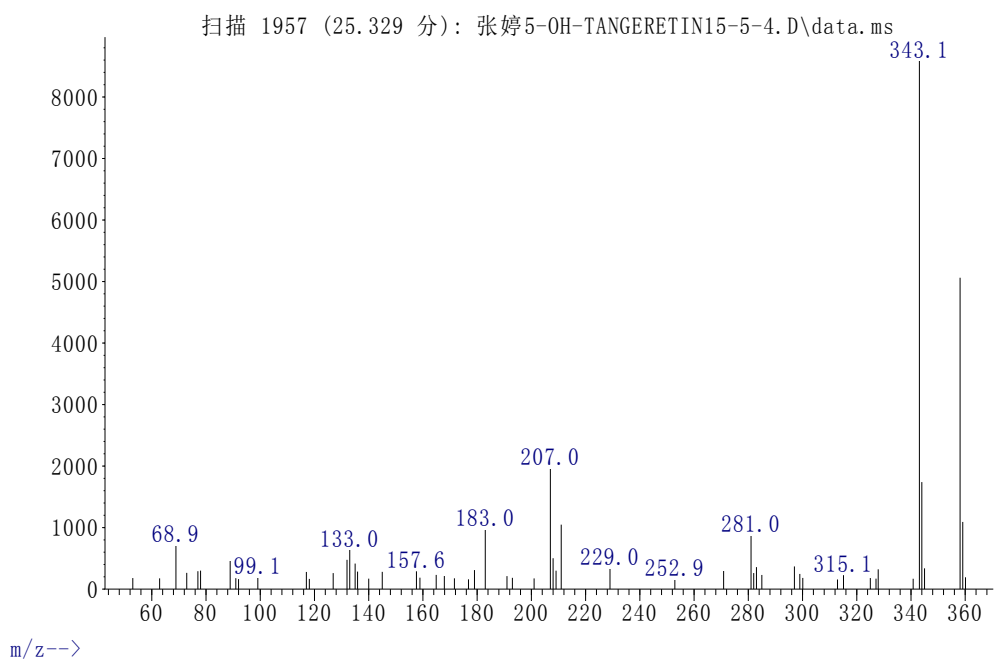
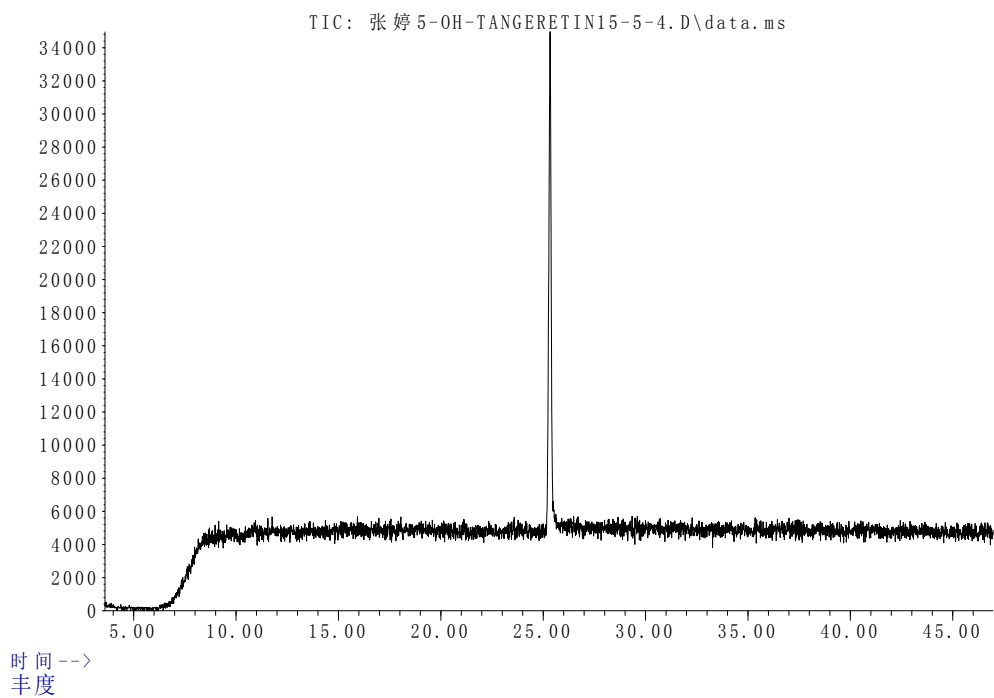


丰度



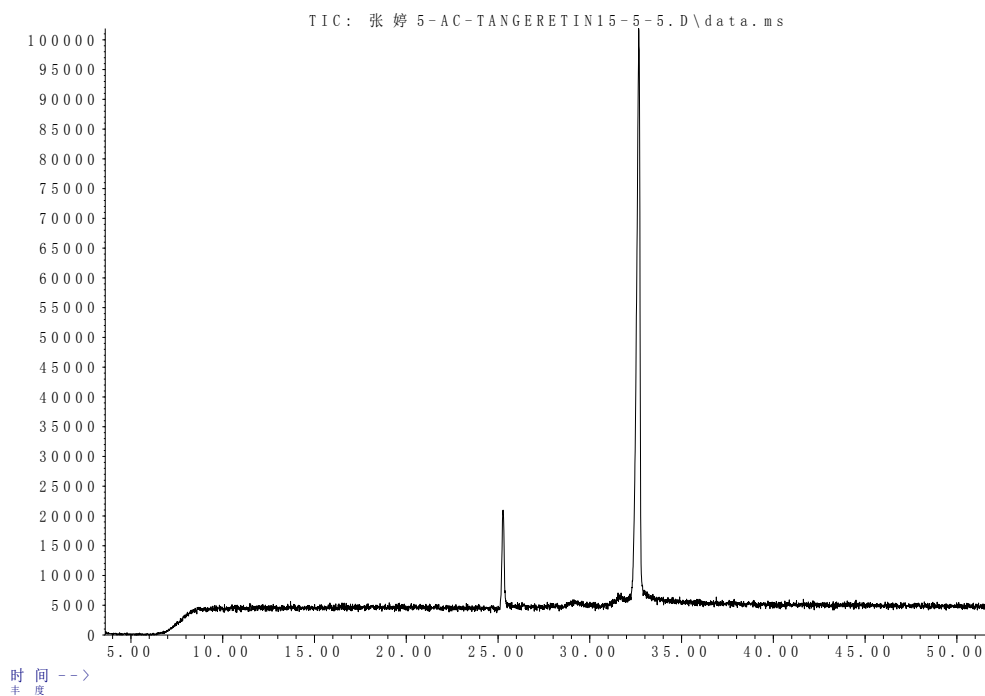
## 10). 5-OH-TAN

丰度

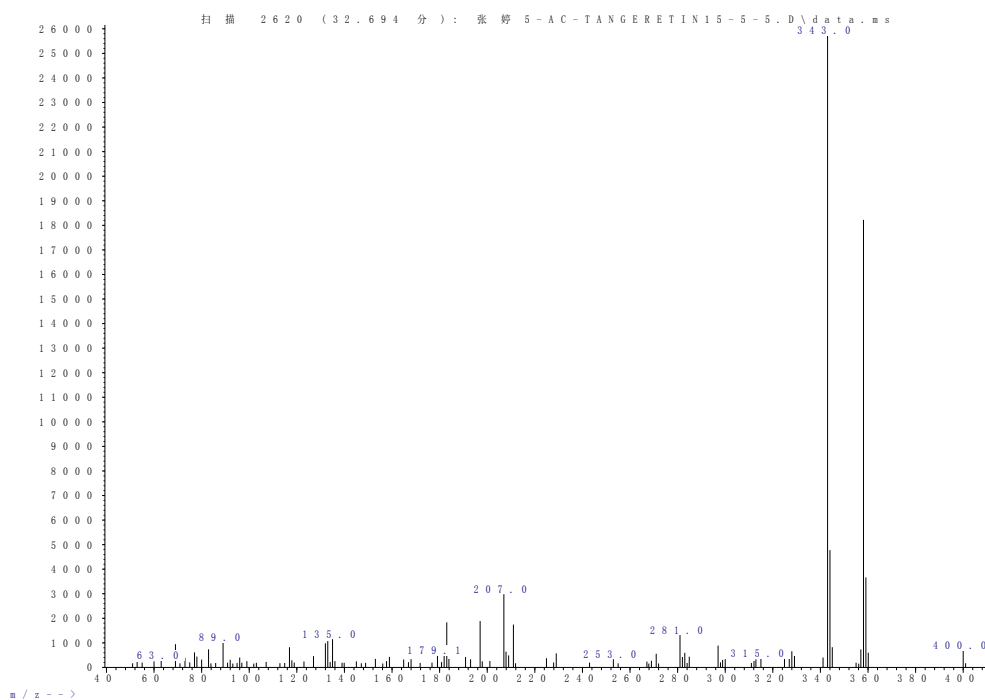


## 11). 5-Ac-TAN

丰度

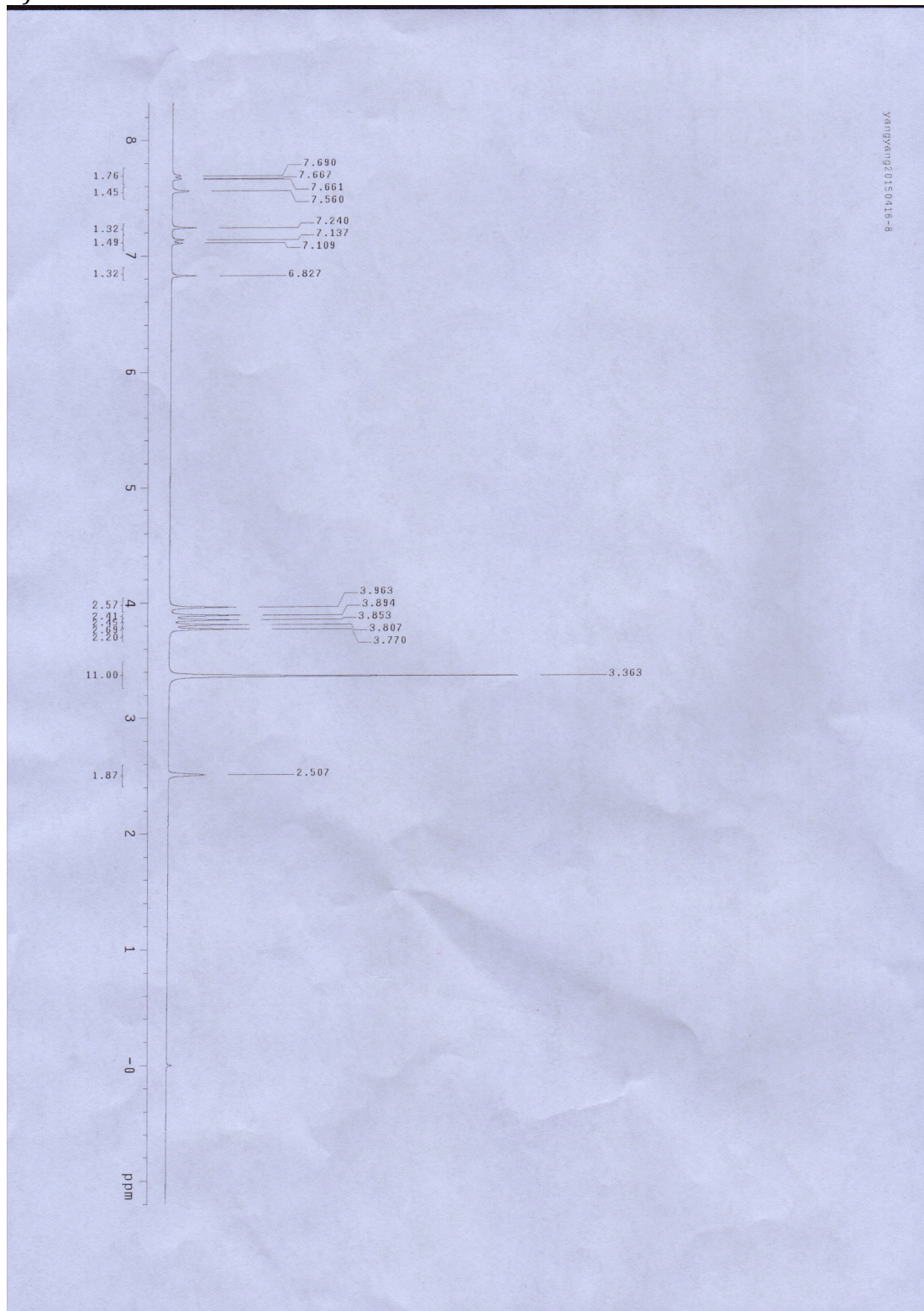


丰度



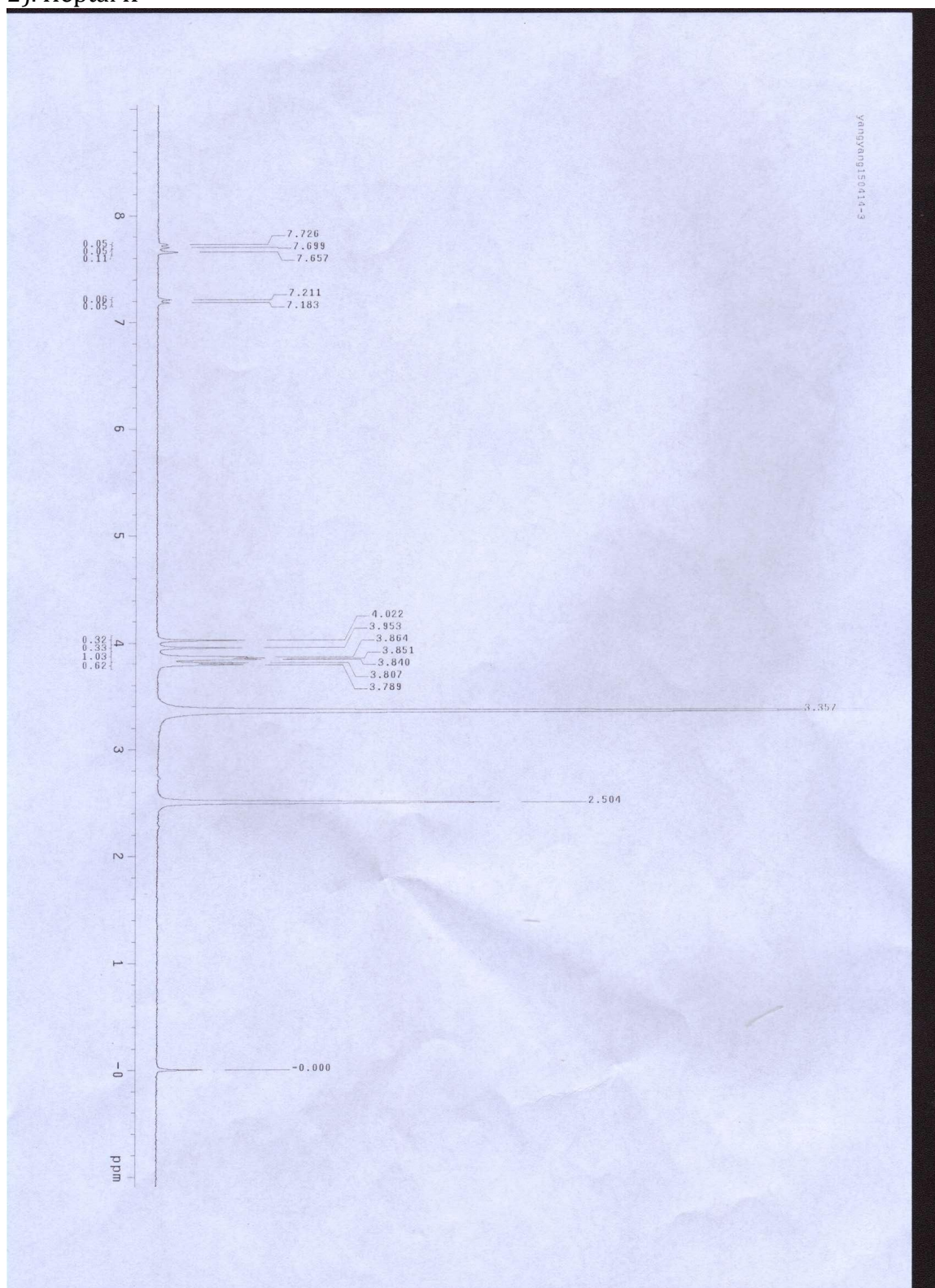
## Appendix B – NMR Graphs

### 1). SIN





## 2). HeptaMF

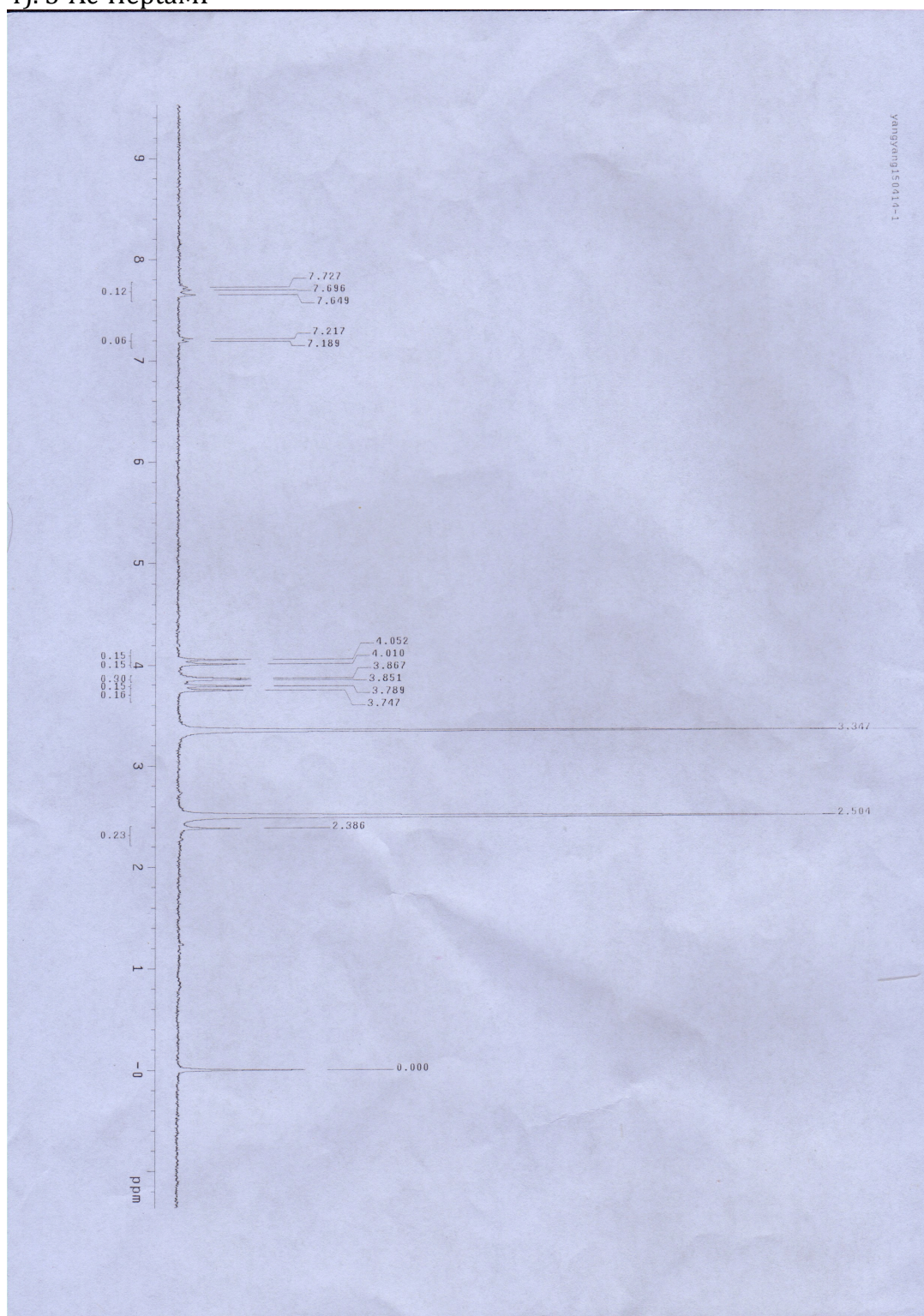




### 3). 5-OH-HeptaMF

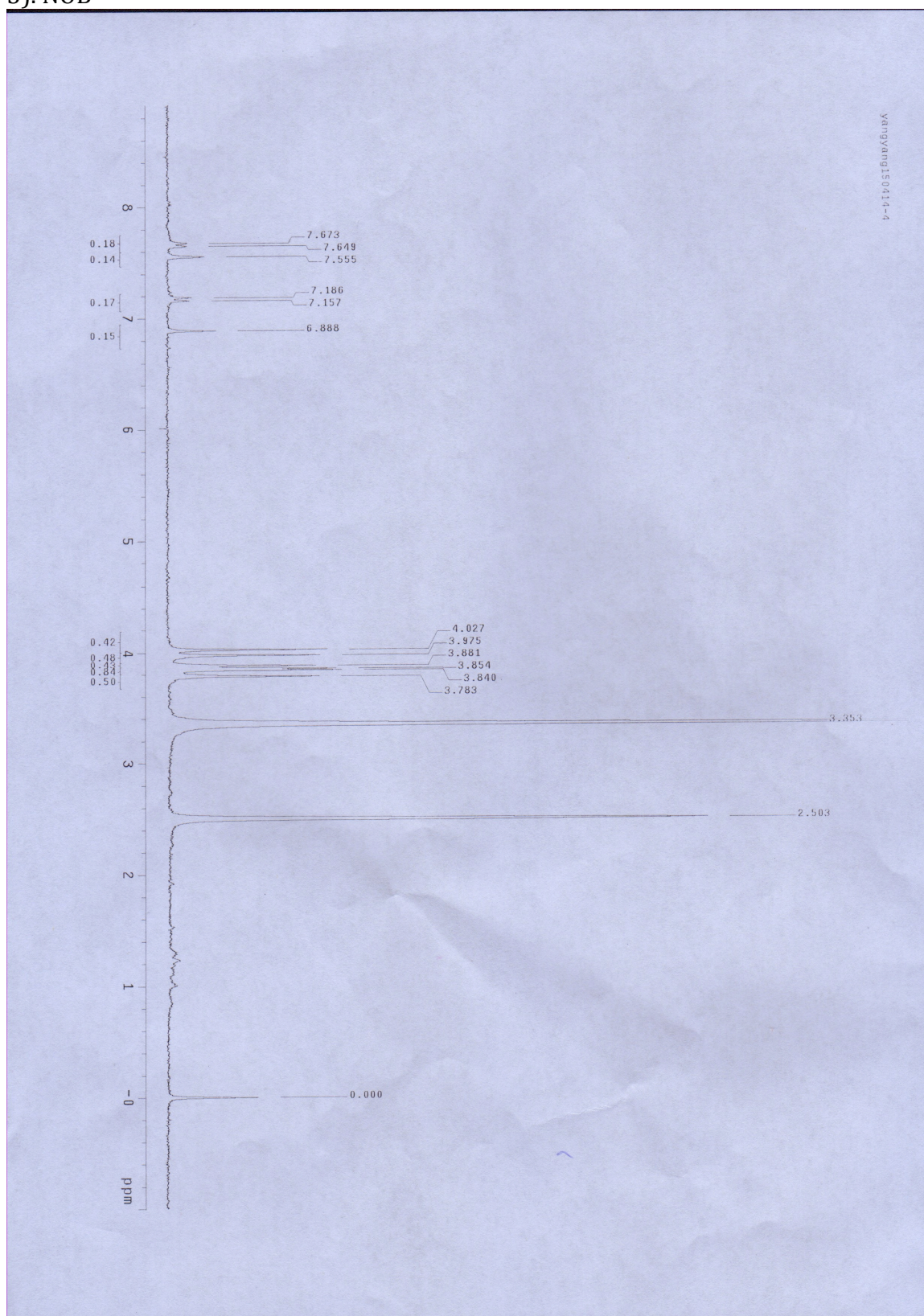


## 4). 5-Ac-HeptaMF



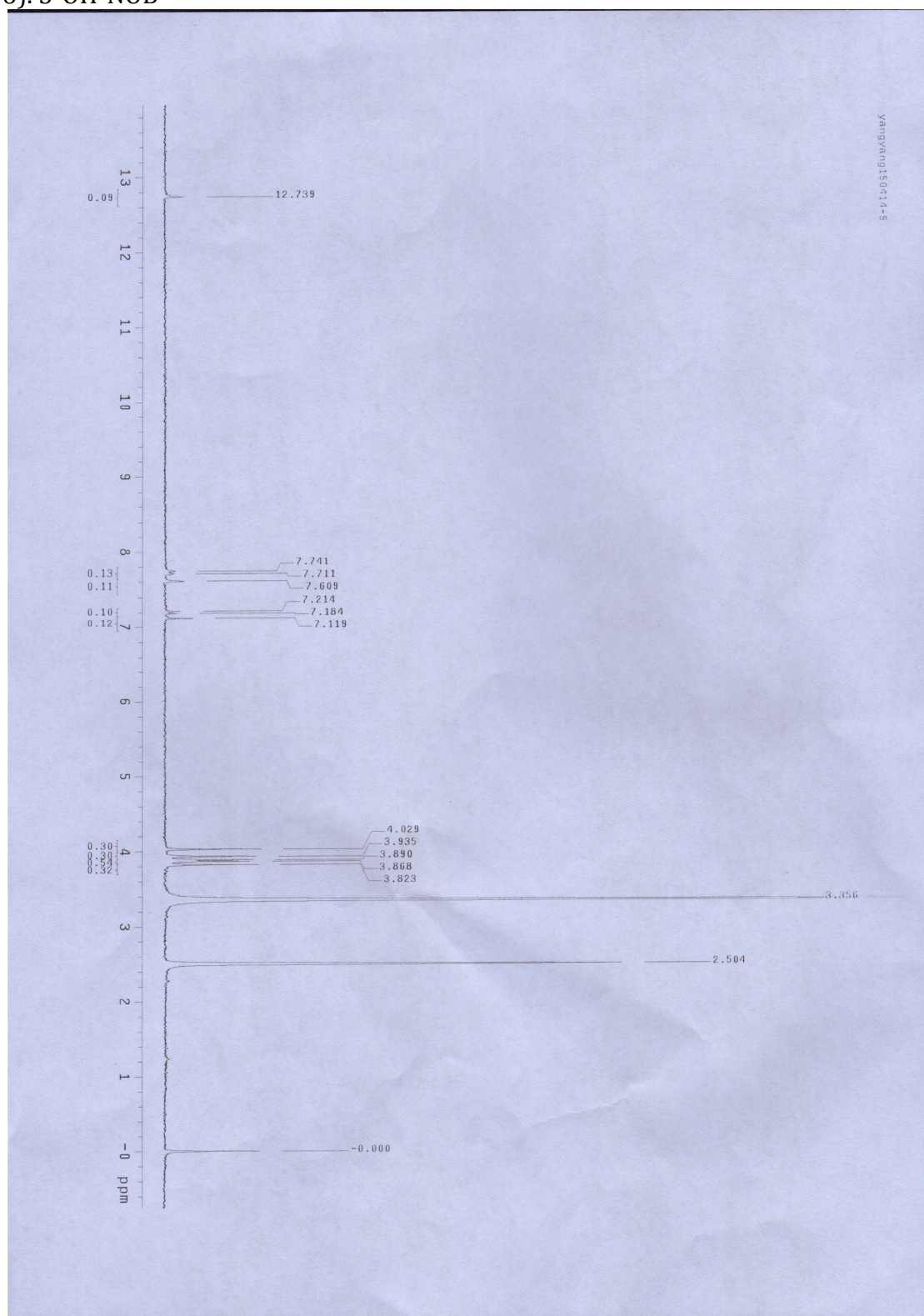


## 5). NOB



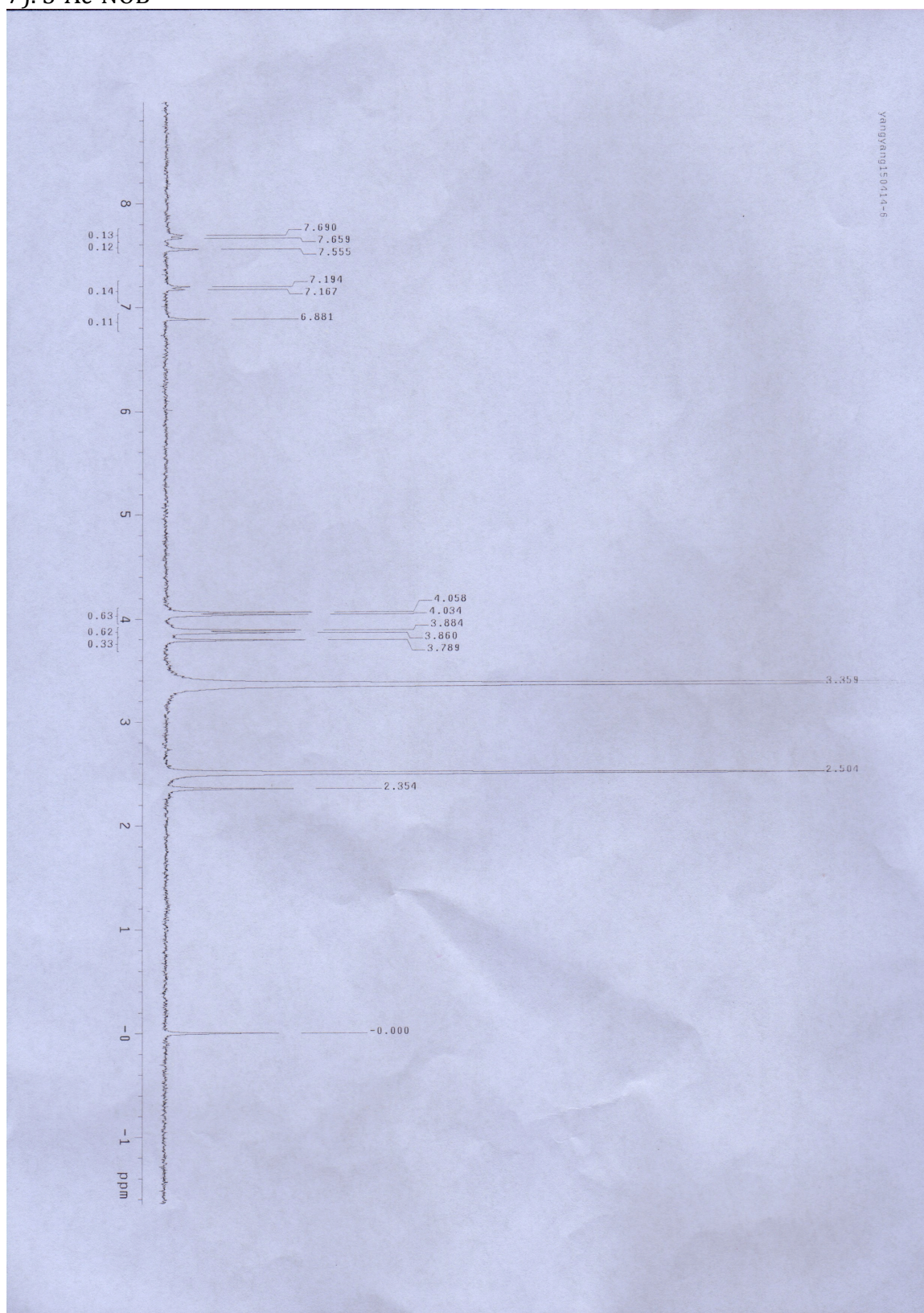


## 6). 5-OH-NOB



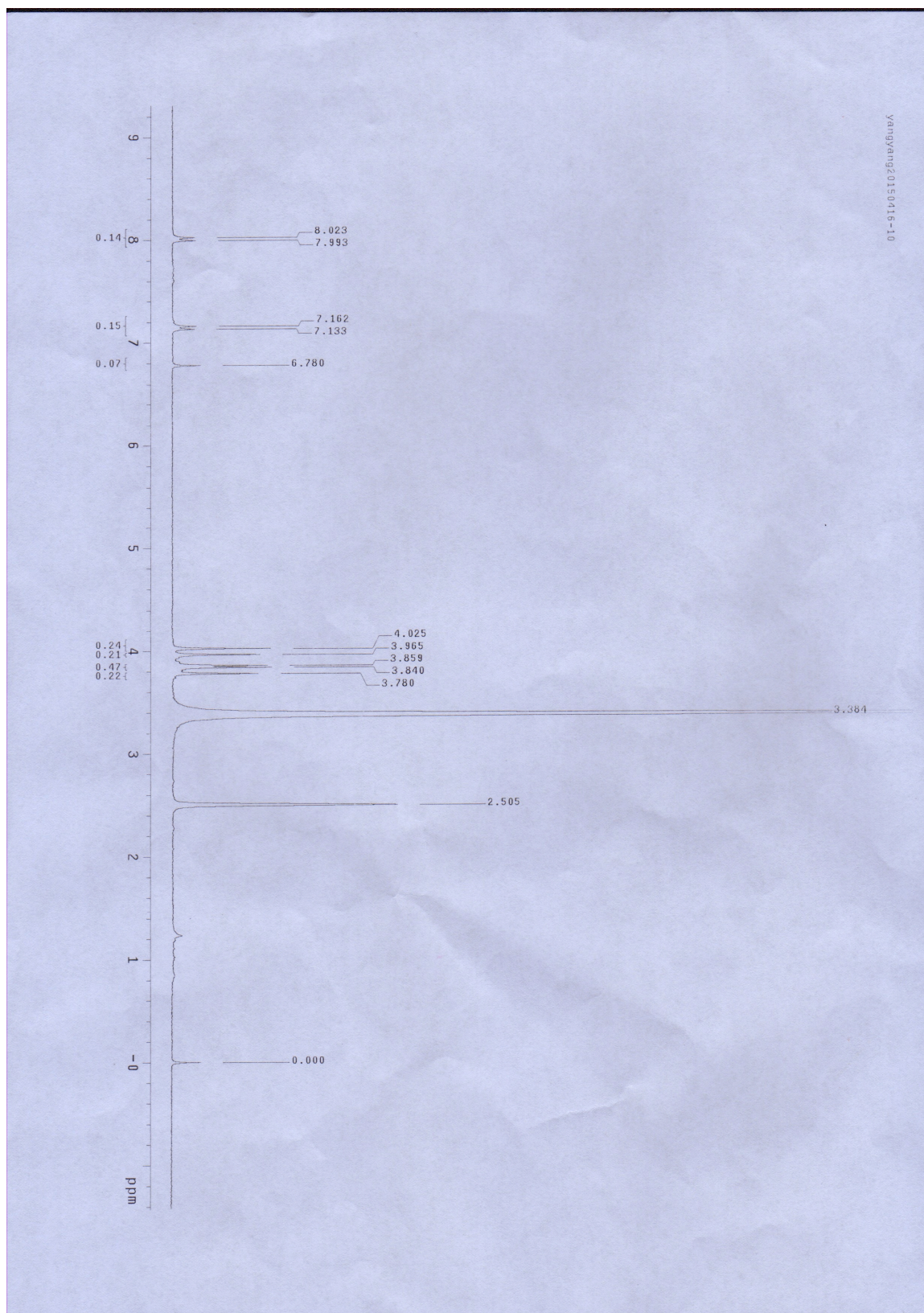


## 7). 5-Ac-NOB



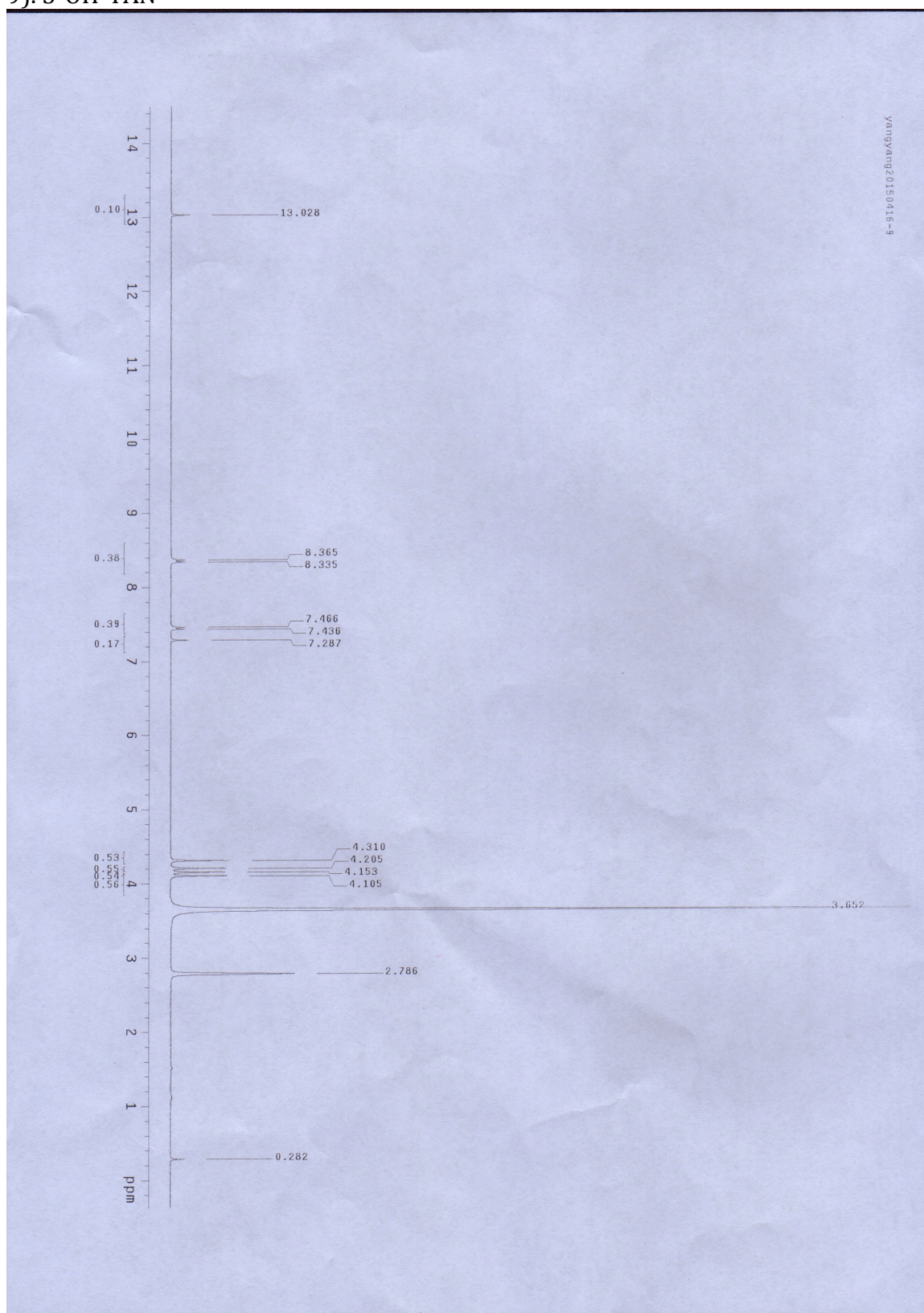


## 8). TAN





## 9). 5-OH-TAN





## 10). 5-Ac-TAN

